Charles University in Prague

Faculty of Science

Study program: Biochemistry

Field of study: Biochemistry

Mgr. Alicja Barbara Veselá

Study of function and molecular architecture of fungal nitrilases applicable in biocatalysis

Dissertation

Supervisor: Ing. Ludmila Martínková, CSc.

Prague, 2015

Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Biochemie

Studijní obor: Biochemie

Mgr. Alicja Barbara Veselá

Studium funkce a molekulární architektury fungálních nitrilas využitelných v biokatalýze

Disertační práce

Školitel: Ing. Ludmila Martínková, CSc.

Praha, 2015

I hereby declare that this thesis is based on my own research carried out in the Laboratory of Biotransformation, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., except where due acknowledgement has been made in the text, and that all sources of information are cited. No part of the work was used for obtaining the same or different academic title.

Prague, August 21, 2015

Signature

Acknowledgements

I would like to thank my supervisor, Ing. Ludmila Martínková, CSc. and the head of the department, Prof. Ing. Vladimír Křen, DrSc. for their continuous support and scientific advise throughout my stay at the Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, where this thesis was worked out. I would also like to thank all my colleagues and friends at the laboratory, especially Ing. Ondřej Kaplan, PhD., Mgr. Alena Křenková, PhD., Ing. Anna Rinágelová, Mgr. Petr Marhol, Dr. sc. nat. Michael Kotík, PhD., Mgr. Martin Chmátal CSc. and Mgr. Eva Böhmová for their help and support and for creating a friendly environment.

Special thanks belong to Dipl.-Ing. Birgit Wilding, PhD. and Ao.Univ.-Prof. Dr. Norbert Klempier, PhD. (Institute of Organic Chemistry, Graz University of Technology) for providing me with the taxol precursor substrates and for sharing their knowledge and experience in this field and for the effort put in the manuscript preparation, to Dr. Natallia Kulik, PhD. (Department of Structure and Function of Proteins, Institute of Nanobiology and Structural Biology of GCRC ASCR) for creating the homology models, to RNDr. Oldřich Benada, CSc. (Laboratory of Molecular Structure Characterization, Institute of Microbiology, ASCR) for the electron microscopy analysis and to RNDr. Ondřej Vaněk, PhD. (Department of Biochemistry, Faculty of Science, Charles University in Prague) for the sedimentation velocity analysis.

Finally, I would like to thank my husband and family for their support, patience and understanding.

The work was supported through the following grants:

Czech Science Foundation (No. P504/11/034)

Technology Agency of the Czech Republic (grant no. TA01021368)

IAA500200708 (Grant Agency of the Academy of Sciences of the Czech Republic)

OC09046 (Ministry of Education of the Czech Republic)

COST/ESF CM0701

Abstract

Nitrilases are enzymes which catalyze the hydrolysis of a nitrile into the corresponding carboxylic acid and ammonia. These enzymes are potentially applicable in biocatalysis and bioremediation because of their advantages over the conventional (chemical) methods of nitrile hydrolysis (lower demand for energy, safety, simplicity, high yields, selectivity).

In this work, genome mining was used to search for the sequences of hypothetical nitrilases from filamentous fungi. The amino acid sequences of previously characterized fungal nitrilases were used as the templates. Then the new synthetic genes together with other genes from our nitrilase library were expressed in *E. coli* and the substrate specificities of the enzymes thus produced were compared. Significant attention was focused on the relationships between the sequence of the enzyme and its substrate specificity.

The arylacetonitrilases from *Arthroderma benhamiae* (NitAb) and *Nectria haematococca* (NitNh) were purified and characterized. Their substrate specificities, kinetic parameters, pH and temperature profiles and subunit and holoenzyme size were assessed.

NitAb and NitNh together with other recombinant fungal nitrilases were employed in the hydrolysis of high concentrations of (*R,S*)-mandelonitrile in a batch or fed-batch mode. Nitrilase from *Aspergillus niger* displayed the best results in enantioselectivity, enabling to prepare (*R*)-mandelic acid with 97.6 % e.e., and in catalyst productivity of 40 g of the product per g of dry cell weight. NitAb displayed a moderate enantioselectivity, which, together with its stability at low pH, make it applicable in the production of (*S*)-mandelic acid from (*S*)-mandelonitrile.

A set of recombinant fungal arylacetonitrilases was tested in hydrolysis of (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile. The corresponding carboxylic acid is a precursor of taxol, an anti-cancer drug. All tested enzymes displayed a complete conversion of 1 mM substrate within 1-22 hours. Nitrilase from *Neurospora crassa* was the most active and was thus used for the preparative-scale synthesis of (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carboxylic acid.

Abstrakt

Nitrilasy jsou enzymy katalyzující hydrolýzu nitrilů na příslušné karboxylové kyseliny a amonné ionty. Tyto enzymy mohou nalézt využití v biokatalýze a bioremediaci pro vyšší nenáročnost, bezpečnost a jednoduchost takto katalyzovaných reakcí před konvenčními metodami hydrolýzy nitrilů.

V této práci byly pomocí metody prohledávání databází vybrány sekvence hypotetických fungálních nitrilas. Jako templáty posloužily aminokyselinové sekvence fungálních nitrilas již dříve charakterizovaných v naší laboratoři. Následně byly nové syntetické geny spolu se zbylými geny z naší nitrilasové knihovny exprimovány v *E. coli* a poté byly porovnány jejich substrátové specifity a podobnost sekvencí.

Arylacetonitrilasy z hub *Arthroderma benhamiae* (NitAb) a *Nectria haematococca* (NitNh) byly purifikovány a charakterizovány; byly stanoveny jejich substrátové specifity, kinetické parametry, pH a teplotní profily a velikost podjednotek.

NitAb a NitNh spolu s dalšími rekombinantními fungálními nitrilasami byly použity pro hydrolýzu vysokých koncentrací (*R,S*)-mandelonitrilu v jedné dávce nebo s postupným dávkováním substrátu. Ze studovaných enzymů nitrilasa z *Aspergillus niger* vykázala nejlepší enantioselektivitu (e.e. pro (*R*)-mandlovou kyselinu až 97,6 %) a produktivitu enzymu v sušině (až 40 g $g_{suš}^{-1}$).

NitAb byla pouze mírně enantioselektivní, čehož se však dá v kombinaci s její stabilitou pri nízkém pH využít pro produkci (*S*)-mandlové kyseliny.

Soubor našich rekombinantních fungálních arylacetonitrilas byl rovněž testován na schopnost hydrolyzovat (±)-*trans*-2,4-difenyl-4,5-dihydrooxazol-5-karbonitril (příslušná karboxyová kyselina je prekurzorem taxolu, jež se používá jako protinádorové léčivo). Všechny enzymy dosáhly plné konverze 1 mM substrátu během 1 nebo 22 hodin. Nitrilasa z houby *Neurospora crassa* byla nejvíce aktivní a byla použita při preparativní přípravě (±)-*trans*-2,4-difenyl-4,5-dihydrooxazol-5-karboxylové kyseliny.

List of content

Abbreviations

AA	amino acid
AlaCN	*β*-cyano-L-alanine
ASCR	Academy of Sciences of the Czech Republic
CDH	cyanide dihydratase
CG(s)	cyanogenic glycoside(s)
CHT	cyanide hydratase
CN	nitrile group
CLEAs	cross-linked enzyme aggregates
Cys	cysteine
dcw	dry cell weight
e.e.	enantiomeric excess
GCRC	Global Change Research Centre
Glu	glutamic acid
HCN	hydrogen cyanide
IAA	indole-3-acetic acid
IAN	indole-3-acetonitrile
IPTG	isopropyl β-D-1-thiogalactopyranoside
KCN	potassium cyanide
LB	Luria-Bertani
Lys	lysine
MA	mandelic acid
NHase	nitrile hydratase
NitAb	Nitrilase from *Arthroderma benhamiae* CBS 112371 (GenBank: EFE30690)
NitAn1	Nitrilase from *Aspergillus niger* K10 (CCF 3411) (GenBank: ABX75546)
NitAn2	Nitrilase from *Aspergillus niger* CBS 513.88 (GenBank: CAK46742)

NitAn3	Nitrilase from *Aspergillus niger* CBS 513.88 (GenBank: CAK47246)
NitAo	Nitrilase from *Aspergillus oryzae* RIB40 (GenBank: BAE63579)
NitGm	Nitrilase from *Giberella moniliformis* (GenBank: ABF83489)
NitMg	Nitrilase from *Meyerozyma guilliermondi* (*Pichia guilliermondi*) ATCC 6260 (NCBI Reference Sequence: XP_001482890)
NitNc	Nitrilase from *Neurospora crassa* OR74A (GenBank: CAD70472)
NitNh	Nitrilase from *Nectria haematococca* mpVI 77-13-4 (GenBank: EEU45207)
NitPc1	Nitrilase from *Penicillium chrysogenum* Wisconsin 54-1255 (NCBI Reference Sequence: XP_002562104)
NitPc2	Nitrilase from *Penicillium chrysogenum* Wisconsin 54-1255 (NCBI Reference Sequence: XP_002565836)
NitPm	Nitrilase from *Penicillium marneffei* ATCC18224 (NCBI Reference Sequence: XP_002144951)¨

1 Introduction

1.1 Nitriles and cyanides

Inorganic cyanide and nitrile compounds are widespread in the environment, mainly as a result of human activity but also through cyanide synthesis by a range of organisms including higher plants, fungi and bacteria. The major source of these compounds in soil and water is the discharge of cyanide- and nitrile-containing wastewaters (Baxter and Cummings, 2006). Various nitrile compounds are extensively used as solvents, extractants, pharmaceuticals, feedstock, drug intermediates or pesticides. Nitriles are also important intermediates in the organic synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, heterocyclic compounds and also polymers (Banerjee *et al.*, 2002).

Some nitrile compounds are toxic and mutagenic (Gong *et al.*, 2012). Cyanide is highly toxic to aerobic organisms because it inactivates the respiration system by tightly binding to cytochrome c oxidase (Jandhyala *et al.*, 2003).

1.1.1 Occurrence in nature

Cyanide and various nitriles have been detected in about 3, 000 species of microorganisms, fungi, plants and animals (Gupta *et al.*, 2010). The compounds include cyanogenic glycosides, cyanolipids, *β*-cyano-L-alanine, ricinine (3-cyano-4-methoxy-N-methyl-2-pyridone), phenylacetonitrile, indole-3-acetonitrile and many others (see Fig. 1 for structures).

The most abundant group of naturally occurring cyanide compounds are cyanogenic glycosides (or cyanoglycosides, CGs) (Banerjee *et al.*, 2002). They are produced by ca. 2, 500 plant species termed cyanogenic plants, including several economically important and edible ones, for instance cassava, sorghum, plants of the genus *Prunus*, lima bean and bamboo. CGs are products of plant secondary metabolism, are amino acid-derived and contain an α-hydroxynitrile type aglycon and a

sugar moiety. The aglycons can be divided into aliphatic and aromatic compounds; the sugar is usually D-glucose. (O'Reilly and Turner, 2003; NZ Food Safety Authority: Cyanogenic glycosides – Information sheet; Vetter, 2000).

The CGs are stored in the vacuoles and their function in the plants is mainly the defense against herbivores and phytopathogens, but in some cases they also serve for storing sugars and reduced nitrogen (Zagrobelny *et al.*, 2008). When the plant is wounded, CGs are metabolized, which leads to hydrogen cyanide (HCN) liberation (Zagrobelny *et al.*, 2010; Vetter, 2000). This enzymatic pathway leading to HCN production accounts for the potential toxicity of the cyanogenic plants, some of which serve as food for humans and animals (Conn, 1979).

Apart from plants, CGs were also found in arthropods, namely within *Diplopoda*, *Chilopoda* and *Insecta.* Also in this case, the CGs serve as a means of defense against predators, and are present in the defensive secretions (Duffey, 1981; Zagrobelny *et al.*, 2010).

Cyanolipids, which are exclusively found in plants, are esters of α- or γ-hydroxynitriles and fatty acids. They share a structural similarity with CGs, however, they are not nearly as abundant, although it was documented that cyanolipids and CGs might be biosynthetically connected (Bjarnholt and Møller, 2008).

β-Cyano-L-alanine (AlaCN) is widespread in higher plants as a metabolite of HCN (Piotrowski *et al.*, 2001). Apart from plants, this compound was also observed to be produced by various bacterial strains (*Escherichia coli*, *Bacillus megaterium*, *Chromobacterium violaceum* and *Enterobacter* sp. 10-1) grown on a nitrile-containing medium (Legras *et al.*, 1990).

Indole-3-acetonitrile (IAN) and phenylacetonitrile are nitrile derivatives of plant hormones (auxins) and in some cases they were demonstrated to have auxin activity of their own (Piotrowski 2008).

Cyanide compounds such as glyoxylic acid cyanohydrin, pyruvic acid cyanohydrin and several α-aminonitriles have been also reported in a number of fungi (Tapper and McDonald, 1974; Akken and Strobel, 1966; Faull *et al.*, 1994) and bacteria (Kikuchi, 1955; Parker *et al.* 1988). The toxicity of these compounds serves to inhibit competitive organisms (Baxter and Cummings, 2006).

As for the animals, the ability to synthesize nitriles seems to be restricted to sponges and arthropods. In sponges, the nitrile compounds serve as antimicrobial agents; in arthropods, they serve as protection against predators (Davis and Nahrstedt, 1985; Duffey, 1981).

1.1.2 Metabolism of cyanide and nitriles

1.1.2.1 Hydrogen cyanide

Cyanogenesis, the production of HCN, was documented in bacteria, single-cell algae and fungi. In bacteria and fungi, use of labeled compounds showed that the source of HCN was the amino acid glycine; in the alga *Chlorella vulgaris*, the HCN precursor was histidine (Knowles and Bunch, 1986; Gewitz *et al.*, 1976). HCN is also produced in equimolar amount to ethylene by all higher plants during the biosynthesis of the plant hormone ethylene (Peiser *et al.*, 1984; Pirrung, 1985), and as stated before, in plants and arthropods producing CGs, HCN is released upon wounding or in defense against predators.

Natural cyanide degradation can be achieved by four major pathways: oxidative, reductive, substitution/transfer and hydrolysis. Several organisms have been reported to use more than one of these pathways. It strongly depends on the external conditions, which one of the pathways is followed (Ebbs, 2004).

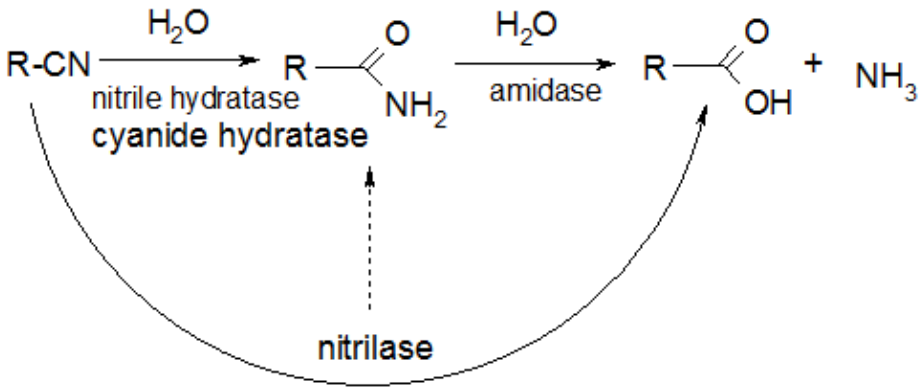
The hydrolytic pathway of cyanide biodegradation involves cyanide hydratases (CHTs) and cyanide dihydratases (CDHs). CHTs convert cyanide into formamide, CDHs catalyze the hydrolysis of HCN to formic acid ad ammonia. Further information on both CHTs and CDHs will be given in Sections 1.2.3 and 1.2.4.

1.1.2.2 Nitriles

Nitriles can be naturally degraded in several pathways. In plants, fungi and insects, oxidation of some nitriles is catalyzed by an oxygenase. The resulting

cyanhydrin decomposes to an aldehyde and HCN either spontaneously or by action of a hydroxynitrile lyase (Legras *et al.*, 1990).

Enzymatic hydrolysis is the most explored of the nitrile biodegradation routes. It can be achieved in one step, catalyzed by nitrilase, or in a two-step reaction, catalyzed by nitrile hydratase and amidase (Fig. 1.1).



Cyanide hydratase: R = H

Nitrilase, nitrile hydratase: R = alkyl, aryl

Figure 1.1: Scheme of enzymatic nitrile hydrolysis

According to their sequence and functional similarities, some amidases and nitrilases are members of the nitrilase superfamily. Nitrilases form branch 1 together with the CHTs and CDHs; amidases form next two branches (O'Reilly and Turner, 2003; Brenner, 2002). More details on these enzymes will be given in the next Section.

1.2 Enzymes of nitrile and cyanide hydrolysis

1.2.1 Nitrile hydratases

Nitrile hydratase (NHase) catalyzes the hydration of the nitrile moiety into an amide. It is not, however, a member of the nitrilase superfamily of enzymes, and the type of catalyzed reaction defines NHase as a lyase.

NHases are enzymes with a metal cofactor, and are divided into Fe-type and Co-type enzymes.

These enzymes have been isolated and characterized in a plenty of microorganisms, for instance *Agrobacterium tumefaciens* (Bauer *et al.*, 1994), *Pseudomonas putida* (Payne *et al.*, 1997), *Corynebacterium* (Tani *et al.*, 1989), and various bacteria of the genus *Rhodococcus* (Endo and Watanabe 1989; Nagasawa *et al.*, 1991; Duran *et al.*, 1993; Kato *et al.*, 1999; Přepechalová *et al.*, 2001).

Various NHases prefer different types of substrate – either aliphatic, alicyclic, arylaliphatic, or (hetero)aromatic – but generally the activity is restricted to smaller molecules that do not exceed the volume of the binding pocket (Mylerová and Martínková, 2003).

As for the practical applications of these enzymes, a number of NHase-producing strains have been employed in semi-preparative, preparative and even industrial-scale biocoversions. *Rhodococcus* sp. N774, *Rhodococcus rhodochrous* J1, *Rhodococcus rhodochrous* PA-34, *Pseudomonas chlororaphis* B23 and *Brevibacterium* sp. CH2 have been used for bench or industrial scale production of acrylamide, nicotinamide, isonicotinamide, picolinamide, butyramide, indole-3-acetamide and many other compounds (Prasad and Bhalla, 2010). NHase in *Rhodococcus rhodochrous* IFO 15564 coupled with a non-enzymatic reaction enabled "two-step-one-pot" synthesis of various amides from aldehydes (Kashiwagi *et al.*, 2004). NHase in *Rhodococcus erythropolis* A4 catalyzed the regioselective production of cyanoamides from dinitriles (Vejvoda *et al.*, 2007).

Furthermore, *Rhodococcus* sp. N774, *P. chlororaphis* B23 and *R. rhodochrous* J1 have been successfully adopted in kiloton production of acrylamide and were the first

examples of biocatalysts applied in the industrial manufacture of commodity chemicals (Kobayashi and Shimizu, 1998; Mylerová and Martínková, 2003; Prasad and Bhalla, 2010).

After the initial success of NHases in the production of achiral amides, the focus shifted also to their potential in the synthesis of enatiopure chemicals. Actually, several enatioselective NHases have been described that facilitate the synthesis of (*S*)-arylpropionamides or (*S*)-arylbutyramides (Martínková and Křen, 2002).

1.2.2 Amidases

Amidases are ubiquitous enzymes found in bacteria, fungi, plants and animals. They generally catalyze the hydrolysis of an amide into the corresponding carboxylic acid and ammonia, however, other types of reactions have also been reported. Amidases (except the GGSS signature amidases) belong to the nitrilase superfamily of enzymes, and are sorted into two branches: branch 2 (aliphatic amidases) and branch 3 (N-terminal amidases). The latter catalyze the hydrolysis of an asparagine or glutamine residue at the N-terminus of a polypeptide chain into aspartic or glutamic acid (Fournand and Arnaud, 2001; Brenner, 2002).

Apart from the amide hydrolysis, the acyl-transfer reaction in the presence of hydroxylamine and the resulting formation of a hydroxamic acid is a well-described phenomenon for amidases, and a broad range of hydroxamates have been prepared in this way (Maestracci *et al.*, 1986; Fournand *et al.*, 1998; Vejvoda *et al.*, 2011; Bhatia *et al.*, 2013).

The aliphatic amidases are highly stereoselective towards substrates with a center of chirality at the α-position and have a potential for large-scale production of compounds such as amino acids and 2-arylpropionic acids, valuable chemicals for the pharmaceutical industry. An example of the industrial-scale amidase-catalyzed reaction is the chiral resolution of racemic 2,2-dimethylcyclopropane carboxamide affording the optically pure *S*-isomer (Shaw *et al.*, 2003).

In many cases, whole-cell preparations of NHase and amidase-producing bacteria were employed in the synthesis of various optically pure amides and carboxylic

acids, for instance (*R*)-α-substituted arylalkanamides and (*S*)-α-substituted arylalkanoic acids (Wu and Li, 2001), (*S*)-2-aryl-3-methylbutyric acids and (*R*)-2-aryl-3-methyl-butyramides (Wang *et al.*, 2001), γ-substituted α-methylene carboxamides and acids (Wang and Wu, 2003), or enantiopure *trans*- and *cis*-cyclopropanecarboxamides and acids (Wang and Feng, 2002; Wang *et al.*, 2004).

NHase- and amidase-producing bacterial strains can be also applied in the bioremediation of nitrile-contaminated sites (Kohyama *et al.*, 2006; Li *et al.*, 2007; Baxter *et al.*, 2006). For instance, benzonitrile herbicides dichlobenil and bromoxynil were converted to the corresponding amides and acids by the whole-cell suspension of *Rhodococcus erythropolis* A4 (Veselá *et al.*, 2012).

1.2.3 Branch 1 of the nitrilase superfamily

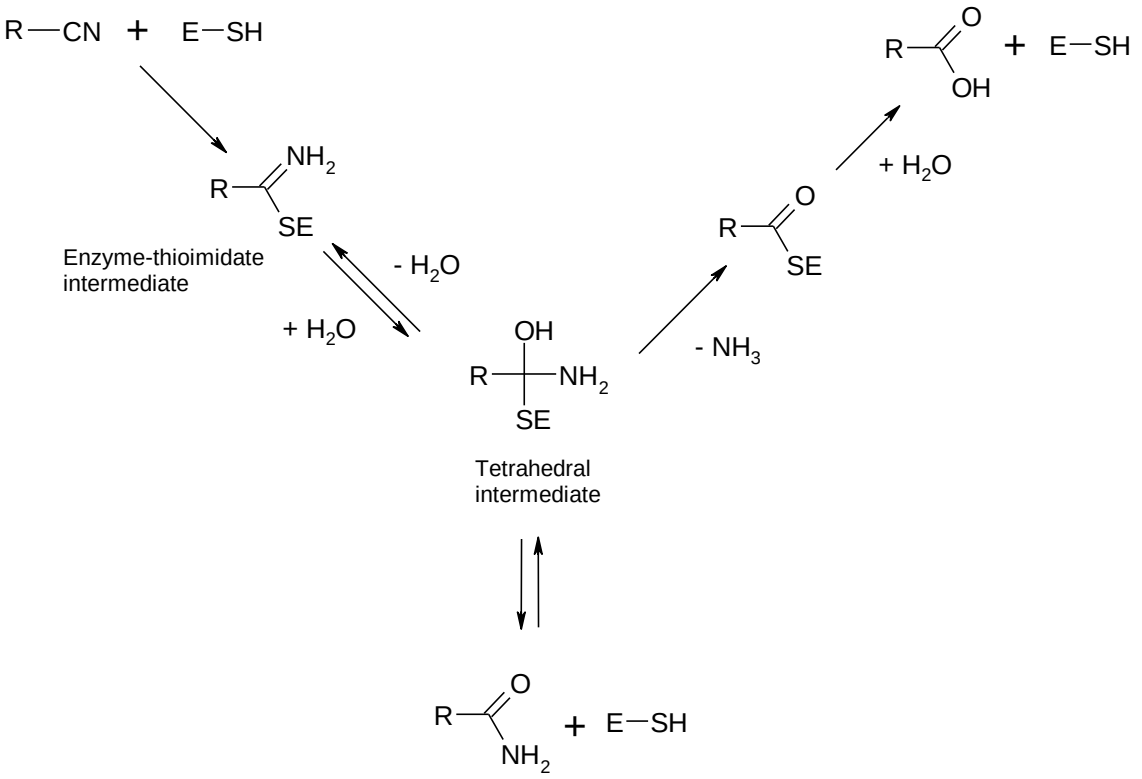
Cyanide hydratases, cyanide dihydratases and nitrilases belong to branch 1 of the nitrilase superfamily of enzymes (formerly called CN-hydrolases; O'Reilly and Turner, 2003). Nitrilase superfamily comprises thiol enzymes involved in natural product biosynthesis and posttranslational modification in prokaryotes, fungi, plants and animals. The superfamily is divided into 13 branches, but only branch 1, also called nitrilase family, exhibits the nitrile-hydrolyzing activity (Pace and Brenner, 2001).

The common features of all three types of the branch 1 enzymes are their molecular architecture, mode of action, and the catalytically active amino acid residues.

The enzyme subunits form a homodimeric building block with a αββα- αββα sandwich fold. The homodimers further assemble into active oligomers made of 4-26 subunits or active spirals of variable length (Thuku *et al.*, 2009).

The catalytic residues in the active center are two glutamic acids, a lysine and a cysteine. The sulfhydryl group of the cysteine residue performs the nucleophilic attack on the CN bond of the nitrile/cyanide to form enzyme-thioimidate intermediate (Fig. 1.2). The following water addition leads to the formation of a tetrahedral intermediate. At this point, the leaving group may be either the enzyme, or ammonia (Stevenson *et al.*, 1992). In general, ammonia is a better leaving group than the enzyme, and thus - in the nitrilases and cyanide dihydratases -the reaction continues with the formation of an

acylenzyme, the hydrolysis of which produces the carboxylic acid product (Brenner, 2002). Cyanide hydratases follow this mechanism only to the tetrahedral intermediate stage. Here the enzyme is the leaving group, and formamide is the final product, possibly because some of the steric requirements of the active site are not met (Pace and Brenner, 2001).



Cyanide hydratase, cyanide dihydratase: R = H

Nitrilase: R = alkyl, aryl

Figure 1.2: The reaction mechanism of nitrilase, cyanide hydratase and cyanide dehydratase. Route leading to amide formation is preferred by cyanide hydratases, whereas the route leading to carboxylic acid and ammonia formation is preferred by nitrilases and cyanide dihydratases (adapted from O'Reilly and Turner, 2003)

1.2.3.1 Cyanide hydratases

Cyanide hydratase (formamide hydrolyase, EC 4.2.1.66, CHT) catalyzes the hydration of cyanide to formamide. Although CHTs are lyases, according to their sequence similarities they belong to the nitrile-hydrolyzing branch 1 of the nitrilase superfamily. Of all the kingdoms of the living organisms, CHTs seem to be restricted to phytopathogenic and saprophytic filamentous fungi, where their role is to detoxify the HCN released by the host plant. To date, CHT have been studied in several native producers and also expressed in heterologous hosts (Tab 1.1). The sequences of the CHTs documented so far share a very high similarity and seem to be closely related to each other (O'Reilly and Turner, 2003).

Table 1.1: Cyanide hydratase producers

Native producer	Ref.
Gloeocercospora sorghi	Fry and Munch, 1975; Wang *et al.*, 1992; Wang and VanEtten, 1992
Fusarium lateritium	Cluness *et al.,* 1993
Fusarium solani	Barclay *et al.,* 1998; Nolan *et al.*, 2003
Fusarium oxysporum	Yanase *et al.*, 2000
Leptosphaeria maculans	Sexton and Howlett 2000
Aspergillus nidulans[a]	Basile *et al.*, 2008
Neurospora crassa[a]	
Gibberella zeae[a]	
Gloeocercospora sorghi[a]	
Aspergillus niger [a]	Rinágelová *et al.*, 2014
Penicillium chrysogenum[a]	Kaplan *et al.*, 2013

[a] Expressed in *E. coli*

The molecular mass of CHT subunits is typically about 40 kDa, and in case of CHTs from *G. sorghi* (Woodward *et al.*, 2008) and *N. crassa* (Dent *et al.*, 2008), the subunits were documented to assemble into long regular helices (Thuku *et al.*, 2009).

Both the specific activity and K_m for HCN in the studied CHTs were high: 10^2–10^3 U mg^{-1} protein and *ca.* 100 mM, respectively (Yanase *et al.*, 2000; Jandhyala *et al.*, 2003; Basile *et al.*, 2008; Rinágelová *et al.*, 2014).

As for the origin of CHTs, it was speculated that the *cht* genes are probably the result of evolution of nitrilase genes acquired by fungi via horizontal gene transfer from bacteria (Podar *et al.*, 2005). This statement is supported by the fact that several of the studied CHTs also displayed nitrilase activities (Tab. 1.2). The nitriles were turned either to carboxylic acids and ammonia, to amides, or to a mixture of acid, amide and ammonia.

Table 1.2: Cyanide hydratases with nitrilase activity

CHT source	Substrates of the nitrilase activity (% of the CHT acitivity)	Ref.
F. lateritium	Benzonitrile, acetonitrile, propionitrile (*ca.* 0.009-0.033)	Nolan *et al.*, 2003
F. oxysporum	Methacrylonitrile, crotononitrile, acrylonitrile (*ca.* 0.03-0.04%)	Yanase *et al.*, 2000
Aspergillus niger	Fumaronitrile (0.95) 2-Cyanopyridine (0.76) Benzonitrile (0.062) 3-Cyanopyridine (0.055)	Rinágelová *et al.*, 2014
Penicillium chrysogenum	Fumaronitrile (1.8) 2-Cyanopyridine (0.83) Benzonitrile (0.042) 3-Cyanopyridine (0.15)	Rinágelová, 2013

Several studies were carried out aiming to propose possible application of various enzyme preparations of CHTs in cyanide bioremediation (Cluness *et al.*, 1993; Basheer *et al.*, 1992; Campos *et al.*, 2006; Ingvorsen *et al.*, 1992; Basile *et al.*, 2008; Martínková *et al.*, 2015).

1.2.3.2 Cyanide dihydratases

Cyanide dihydratase (cyanidase, CDH) catalyzes the hydrolysis of cyanide to formic acid and ammonia. With one exception (Kunz *et al.*, 1994), no formamide has ever been detected in the CDH-catalyzed reactions. So far, CDH were only found in bacteria, namely *Alcaligenes xylosoxidans* ssp. *denitrificans* (Ingvorsen *et al.*, 1991), *Bacillus pumilus* C1 (Meyers *et al.*, 1993), *Pseudomonas fluorescens* NCIMB 11764 (Kunz *et al.*, 1994) and *Pseudomonas stutzeri* AK61 (Watanabe *et al.*, 1998).

CDHs also tend to form oligomeric structures. CDH from *P. stutzeri* was found to be a spiral comprising 14 subunits with 2-fold symmetry (Sewell *et al.*, 2003). At neutral pH, the CDH from *B. pumilus* formed a short, 18-subunit spiral, however, when the pH lowered to 5.4, the enzyme reassembled to long helical fibres (Jandhyala *et al.*, 2003). The functional significance of oligomerization seems to be an increase in activity (Jandhyala *et al.*, 2005, Sewell *et al.*, 2005) and forming of organelle-like structures (Thuku *et al.*, 2007).

Unlike CHTs, the CDHs have a very narrow range of pH optimum and stability (Jandhyala *et al.*, 2005). The CDH from *B. pumilus* displayed a significant loss of activity above pH 8 and no activity above pH 8.4 (Meyers *et al.*, 1993). In order to improve the operational stability of the enzymes or to elucidate the role of the C-terminal region in the enzyme oligomerization and activity, various mutants of CDHs were constructed (Sewell *et al.*, 2005).

B. pumilus CDH and *P. stutzeri* CDH hybrid had an enhanced thermal and pH stability, and it was shown that the C-terminus from *P. stutzeri* stabilizes the new hybrid enzyme by supporting oligomerization between the subunit dimers (Sewell *et al.*, 2005; Crum *et al.*, 2015).

CDHs have been also investigated as means for the cyanide detection in biosensors (Mak *et al.* 2005; Keusgen *et al.* 2004; Ketterer and Keusgen 2010).

1.2.3.4 Nitrilases

Nitrilases (EC 3.5.5.1) catalyze the hydrolysis of nitriles into the corresponding carboxylic acid and ammonia. They are abundant in nature and occur in both prokaryotes and eukaryotes (Thuku *et al.*, 2009). Depending on the preferred substrate structure, nitrilases can be divided into aliphatic, aromatic and arylaliphatic. The last group is especially promising in biocatalysis and chemo-enzymatic synthesis because of the ability to hydrolyze α-substituted arylaliphatic nitriles stereoselectively (Kobayashi and Shimizu, 1994; Martínková and Křen, 2010).

The reaction mechanism is the same as in cyanide dihydratases, only the substrate is not HCN, but a nitrile (Fig. 1.2).

Under specific conditions, amide can be formed as a side-product. This seems to be strongly affected by the nature of the substituent on the α-carbon position. In the nitrilase from *Pseudomonas fluorescens* EBC 191, it was shown that the electron-deficient substituents promoted the amide formation, as well as low temperature and increased pH. The portion of amide was also more significant in the reaction products of the less-preferred substrate enantiomers (Fernandes *et al.*, 2006). The NIT1 enzyme in *Arabidopsis thaliana* also formed amides preferentially from substrates with electron-withdrawing substituents, which are thought to stabilize the tetrahedral intermediate in the reaction (Osswald *et al.*, 2002).

In biocatalytic applications where the acid is the only desired product, the amide formation is usually a drawback. However, it can be also turned into an advantage. Based on mutagenetic studies, it was found that enhanced amide-formation ability of certain nitrilase mutants could be utilized in a biocatalytic production of optically pure amides (Kiziak and Stolz, 2009).

In addition to the occasional amide production, nitrilase from *Rhodococcus rhodochrous* J1 was found to have another activity, that is the ability to catalyze the

hydrolysis of amides to carboxylic acids, but the relative rate of this reaction was six orders of magnitude lower than that of nitrile hydrolysis (Kobayashi *et al.*, 1998).

The subunit size of nitrilases is between 30 – 45 kDa. Although some nitrilases were reported to be active as monomers (Bandyopadhyay *et al.*, 1986; Bhalla *et al.*, 1992) or dimers (Mueller *et al.*, 2006) – the majority of nitrilases studied to date form oligomers or long, helical rods (Fig. 1.3) like the nitrilases from *Fusarium solani* and *Aspergillus niger* K10 (Vejvoda *et al.*, 2008).

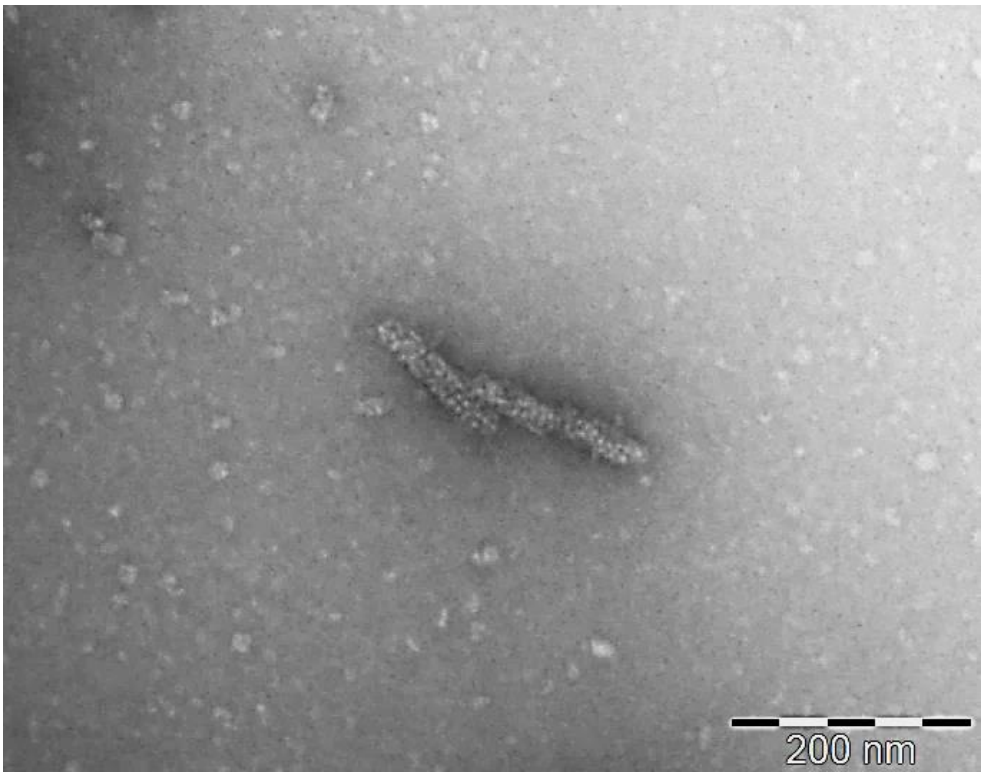


Figure 1.3: Electron micrograph of the rod-like structure of assembled subunits of the nitrilase from *Fusarium solani* (Vejvoda *et al.*, 2008; Author of the micrograph: RNDr. Oldřich Benada, Ph.D., Institute of Microbiology, Academy of Sciences of the Czech Republic)

The enzyme subunits display an αββα-fold, and associate into dimers. The dimers are the building blocks for the oligomerization (Thuku *et al.*, 2009). Furthermore, the subunit association was observed to be induced by the presence of a substrate in the nitrilase from *Nocardia* NCIB 11216 (Harper 1977a), *Rhodococcus* ATCC 39484 (Stevenson *et al.*, 1992), *Alcaligenes faecalis* ATCC8750 (Yamamoto *et al.*, 1992)

and *Rhodococcus rhodochrous* J1 (Nagasawa *et al.*, 2000). The association may be even induced by the addition of ammonium sulphate, organic solvent or on heat treatment (Thuku *et al.*, 2009).

Nitrilases can be either constitutive or inducible and their pH and temperature operational stabilities are within 5.5-11.0 and 10-50°C, respectively. Some nitrilases from thermophilic organisms can be even used at up to 60 or 70°C (Banerjee *et al.*, 2002). The nitrilase-producing microorganisms, fungi and plants, and the characteristics of the isolated enzymes were summarized in several reviews (Banerjee *et al.*, 2002; O'Reilly and Turner, 2003; Thuku *et al.*, 2009; Martínková *et al.*, 2009).

The bacterial nitrilases are the most explored group of these enzymes. Some of these nitrilases, their source organisms, inducers, substrate preferences and activities are summarized in Table 1.3. The most abundant nitrilase source among bacteria is the genus *Rhodococcus*, and except the nitrilase from *R. rhodochrous* K22, which prefers aliphatic substrates, the rhodococcal nitrilases show preference for (hetero)aromatic nitriles. Aliphatic nitrilases were found in *Acidovorax*, *Comamonas*, *Pseudomonas* and *Acinetobacter*. Arylaliphatic nitrilases have been studied in *Pseudomonas*, *Alcaligenes* and *Halomonas* (Martínková and Křen, 2010).

Many of the bacterial nitrilases were expressed in heterologous hosts and a range of mutant variants were prepared (for reviews see Thuku *et al.*, 2009; Martínková and Křen, 2010). Various deletion (Kiziak *et al.*, 2007) and site-specific mutants and chimeric enzymes (Kiziak and Stolz, 2009) were prepared from the arylacetonitrilase of *P. fluorescens* EBC191 and *A. faecalis* ATCC 8750 to test the function of the C-terminal part of the enzyme and the amino acid residues in the proximity of the catalytic cysteine. As a result, amino acid residues responsible for amide formation and enantioselectivity of both enzymes were identified (Kiziak and Stolz, 2009; Sosedov and Stolz, 2014).

There are a large number of nitrile substrates which can be chemo-, regio- and enantioselectively hydrolyzed by nitrilases to the desired carboxylic acid products. Many of the bacterial nitrilases in various preparations have been utilized in such biocatalytic processes, either on laboratory or industrial scale. Such applications were reviewed in several studies (Banerjee *et al.*, 2002; Mylerová and Martínková, 2003;

Singh *et al.*, 2006; Martínková and Křen, 2010; Gong *et al.*, 2012) and some of them will be described in Section 1.3.

Table 1.3 Selected bacterial nitrilases

Source organism	Inducer	Total activity ($U\ L^{-1}$) (substrate)	Ref.
Rhodococcus rhodochrous J1	Isovaleronitrile	16,100 (benzonitrile)	Kobayashi and Shimizu, 1994
R. rhodochrous K22	Isovaleronitrile	1,630 (crotononitrile)	
Rhodococcus sp. NDB 1165	Propionitrile	2,298 (benzonitrile)	Prasad *et al.*, 2007
Alcaligenes faecalis JM3	Isovaleronitrile	4,220 (thiopheneacetontirile)	Nagasawa *et al.*, 1990a
A. faecalis ATCC 8750	*n*-butyronitrile	9.96[a] (mandelonitrile)	Yamamoto *et al.*, 1991
Pseudomonas fluorescens DSM 7155	phenylacetonitrile	5[a] (phenylacetonitrile)	Layh *et al.*, 1998

[a]total activity in U

Nitrilase activity in fungi was already discovered in the beginning of the nitrilase research. Fungal strains of the genera *Fusarium*, *Gibberella*, *Aspergillus*, and *Penicillium* were found to hydrolyze IAN but were not studied any further (Thiman and Mahadevan 1964). Then a nitrilase was purified from *F. solani* IMI196840, a fungus isolated on benzonitrile from the soil of a bromoxynil-treated field (Harper 1977b). A soil isolate, also *F. solani*, degraded the herbicide 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) into several products, two of them being the corresponding carboxylic acid and amide (Hsu and Camper 1978). Whole cells in culture medium and cell-free extract of *Aspergillus fumigatus* were reported to hydrolyze α-aminophenylacetonitrile into *S*-α-phenylglycine, and the hydrolytic activity was attributed to a nitrilase enzyme (Choi and Goo, 1986). Another purified fungal nitrilase was from *F. oxysporum* f. sp. *melonis*. This

enzyme was induced by and hydrolyzed aliphatic and aromatic nitriles (Goldlust and Bohak, 1989). Later, nitrilases from *Aspergillus niger* K10 and *F. solani* O1 were purified and characterized (Kaplan *et al.*, 2006a; Kaplan *et al.*, 2006b; Kaplan *et al.*, 2006c; Vejvoda *et al.*, 2006a; Vejvoda *et al.*, 2006b; Vejvoda *et al.*, 2006c; Vejvoda *et al.*, 2008; Winkler *et al.*, 2009). The fungal nitrilase inducers and enzyme activities are listed in Tab. 1.6.

Table 1.4 Induction and activity of fungal nitrilases produced in native hosts (adapted from Martínková *et al.*, 2009)

Source organism	Inducer	Total activity (U L^{-1})[a]	Ref.
Aspergillus niger K10	3-cyanopyridine	0.2	Kaplan *et al.*, 2006b
	2-cyanopyridine+valeronitrile	170	
Fusarium oxysporum CCF 1414	3-cyanopyridine	1.3	
	2-cyanopyridine+valeronitrile	119.7	
Penicillium multicolor CCF 2244	3-cyanopyridine	0.6	
	2-cyanopyridine	9.3	
Fusarium solani O1	3-cyanopyridine	0.9	
	2-cyanopyridine+valeronitrile	34.0	
	2-cyanopyridine[b]	≥3000	Vejvoda *et al.*, 2006a, Vejvoda *et al.*, 2008
Fusarium solani IMI196840	Benzonitrile	58.7	Harper, 1977b
Fusarium solani f. sp. *melonis*	Acetonitrile	830	Goldlust and Bohak, 1989
	Isobutyronitrile	320	
	Butyronitrile	170	
	Benzonitrile	160	

	Propionitrile	140	

[a] activity determined with benzonitrile

[b] two-stage cultivation

All fungal nitrilases purified from native hosts to date have shown preference for (hetero)aromatic nitriles. Benzonitrile and 3-cyanopyridine act both as preferred substrates and inducers. On the other hand, it was discovered that 2-cyanopyridine is a much more powerful inducer of the nitrilase activity in fungi, despite the fact that it is a very poor substrate for these enzymes (Kaplan *et al.*, 2006b) (See Tab. 1.4).

Only a few fungal nitrilases have been studied in the native producers, despite the fact that protein sequence databases contain a large number of putative fungal nitrilases, most of them in the genera *Aspergillus* and the anamorph-teleomorph pair *Fusarium-Giberella* (Martínková *et al.*, 2009). However, as the number of sequenced genomes has grown and gene synthesis services became more accessible, genome mining as an approach to obtaining new nitrilases turned to be a convenient alternative to the activity-based assays (Seffernick *et al.*, 2009). As a result, sequence-homology based database searches were performed, and several putative fungal nitrilases were expressed in *E. coli* as active enzymes and characterized. Among these new enzymes, not only aromatic nitrilases, but also nitrilases with a strong preference for arylaliphatic substrates were found (Kaplan *et al.*, 2011).

The specific biological roles of nitrilases are not yet fully understood, however, studies have been published on their participation in cyanide detoxification and CG and glucosinolate metabolism in plants. The role in the plant hormone idole-3-acetic acid (IAA) synthesis was proposed, but is still uncertain (Piotrowski, 2008).

The first purified nitrilase enzyme was of a plant origin. The enzyme was isolated from the leaves of barley and was found to catalyze the conversion of indole-3-acetonitrile (IAN) into IAA. The IAA-hydrolysing activity was then found in other 10 plant species from the families *Brassicaceae*, *Poaceae* and *Musaceae*. However, the barley nitrilase accepted also many other nitrile substrates, some with much higher activity than IAN (Thiman and Mahadevan, 1964). Partially purified nitrilase from

chinese cabbage displayed 44 times higher V_{max} for 3-cyanopyridine than for IAN (Rausch and Hildenberg, 1980).

Arabidopsis thaliana produces four nitrilase isoforms (NIT1-4). NIT4 showed a strict preference for AlaCN, with no or trace activities for IAN and other nitrile substrates (Piotrowski *et al.*, 2001). In fact, NIT4 homologs are ubiquitous in higher plants where they participate in cyanide biotransformation by converting AlaCN into asparagine and aspartic acid (Piotrowski and Volmer, 2006).

NIT1-3 share a high degree of sequence identity and are similar in biochemical properties, but differ in their localization in the plant (Vorwerk *et al.* 2001). Homologs of NIT1 were discoverered only in the plant family of *Brassicaceae*, where they seem to have evolved from a NIT4 homolog by gene duplication. Here their primary role is most likely not the synthesis of IAA but rather the participation in the metabolism of glucosinolates (Piotrowski 2008, Janowitz *et al.*, 2009). In *Poaceae*, NIT4 gene duplication probably led to neofuctionalization of the resulting nitrilase isoforms, and their role in the plant is most likely the participation in the metabolism of CGs (Jenrich *et al.*, 2007).

1.3 Nitrilase application in biocatalysis and bioremediation

Enzymatic hydrolysis of nitriles has attracted profound attention since 1980s as it provides a suitable alternative to the conventional methods that use strong acid or base catalysts. Based on their versatility, nitrile-converting enzymes are potentially widely applicable in organic synthesis and present a convenient method to obtain a broad spectrum of useful amides, carboxylic acids, etc. (Brady *et al.*, 2004; Martínková and Křen, 2010; Gong *et al.*, 2012).

Nitrilases have been recognized as valuable biocatalysts for the mild synthesis of high added-value carboxylic acids from cheap and readily available nitriles. Because of their inherent enantioselectivity, nitrilases are also attractive as selective catalysts for setting chiral centers in fine chemical synthesis (Robertson *et al.* 2004). Nitrilases can also discriminate between configurational isomers (*E*,*Z*) (Effenberger and Osswald, 2001). Additionally, they can be used in the synthesis of lactones from 4-hydroxy

nitriles (Taylor *et al.*, 1996), and lactams from α,ω-dinitriles (Gavagan *et al.*, 1998; Brady *et al.*, 2004).

A number of academic institutions and companies (DuPont, Lonza, Dow, Diversa, BASF, DSM; Brady *et al.* 2004) showed interest in exploring the synthetic potential of nitrilases. However, only a few nitrilase-catalyzed processes have been put into practice on industrial or pilot scale so far, like the manufacture of 5-hydroxypyrazine-2-carboxylic acid and 6-hydroxypicolinic acid by Lonza (Liese *et al.* 2000) or (*R*)-mandelic acid and (*R*)-3-chloromandelic acid by Mitsubishi Rayon Co. (Brady *et al.* 2004; Malandra *et al.* 2009). The main reason is probably the relatively low thermostability of the enzyme and the fact that the specific activity and operational stability of most nitrilases is lower than that required in commercial application. Overcoming these drawbacks could further widen nitrilase application in organic synthesis, therefore a number of strategies have been adopted in order to obtain enzymes with new and/or improved properties, like high throughput screening (Black *et al.*, 2015; Coady *et al.*, 2013; Vergne-Vaxelaire *et al.*, 2013; Robertson *et al.*, 2004) , research of extremophiles (Mueller *et al.*, 2006), screening of eukaryotic organisms (Kaplan *et al.*, 2006a), genome mining (Martínková 2014; Gong *et al.*, 2013; Kaplan *et al.*, 2013; Kaplan *et al.*, 2011; Seffernick *et al.*, 2009), rational protein design and directed evolution (Kaul and Asano, 2012; Gong *et al.*, 2012).

Nicotinic acid, (*R*)- and (*S*)-mandelic acid, acrylic acid and glycolic acid production using nitrilases, and the potential of fungal nitrilases in the field of biocatalysis will be discussed in this Section, as well as the role of nitrilases in the biodegradation of nitrile pollutants.

1.3.1 Nicotinic acid

Nicotinic acid (niacin, vitamin B3) a pellagra-preventing factor, is often used in the production of feedstuff additives and pharmaceutical intermediates. Biological route for nicotinic acid production is gaining more attention compared to chemical route as the chemical conversion requires high energy input and separation of the product is very costly with low yield (Almatawah and Cowan, 1999). To date, several

nitrilase-producing organisms have been utilized in the biocatalytic production of nicotinic acid through the hydrolysis of the parent nitrile, 3-cyanopyridine. Tab. 1.5 gives recent examples of these bioconversions.

Table 1.5 Nitrilase-producing organisms in the nicotinic acid production

Organism	Biocatalyst Process mode	Substrate conc. (M)	Productivity	Ref.
Rhodococcus sp. NDB 1165	Resting cells Fed batch	1.6 [a]	8.95 g h^{-1} g_{DCW}^{-1}	Prasad *et al.*, 2007
Nocardia globerula NHB-2	Resting cells Fed batch	0.1	24.6 g h^{-1} g_{DCW}^{-1}	Sharma *et al.*, 2011
Gibberella intermedia CA3-1	Alginate immobilized cells Fed batch	0.2	205.7 g g_{DCW}^{-1} [b]	Li *et al.*, 2015

[a] cumulative concentration

[b] after 28 consecutive batches

Cell free extract from *A. niger* K10 adsorbed onto a 1mL HiTrap Butyl Sepharose column converted of 3-cyanopyridine and 4-cyanopyridine. The initial reaction was nearly quantitative. The conversion of 3-cyanopyridine decreased to 70% after 15 h, the conversion of 4-cyanopyridine decreased to 60% after 39 h. Amide by-product was formed in both cases – the molar ratio of nicotinic acid and nicotinamide was ca. 16:1 and of isonicotinic acid and isonicotinamide ca. 3:1 (Vejvoda *et al.*, 2006a).

Mycelium of *F. solani* O1 entrapped in LentiKats® and the cell-free extract immobilized onto a Butyl Sepharose column hydrolyzed 100 mmol L^{-1} and 10 mmol L^{-1} 3-cyanopyridine, respectively. The entrapped mycelium hydrolyzed 74 % of the substrate within 75 h, the column preparation reached nearly quantitative conversion (>99 %) within 72 h. The conversion of 10 mmol L^{-1} concentration afforded more nicotinamide (ca. 5 % of the total product) than the 100 mmol L^{-1} conversion of the same substrate by the entrapped mycelium (Vejvoda *et al.*, 2006b).

1.3.2 (*R*)- and (*S*)-Mandelic acid and (*R*)-*o*-chloromandelic acid

Enantiomerically pure 2-hydroxycarboxylic acids are valuable synthetic building blocks. (*R*)-Mandelic acid is frequently used as an optical resolving agent and intermediate for the production of various pharmaceutical and agricultural products, and is currently produced through the nitrilase reaction on the scale of several tons per year by BASF (Germany) and Mitsubishi Rayon Japan (Gong *et al.*, 2012). A dynamic kinetic resolution (Fig. 14) of the chemically synthesized cyanohydrin in the presence of an enantioselective nitrilase affords the (*R*)-acids (Rantwijk and Stolz, 2015).

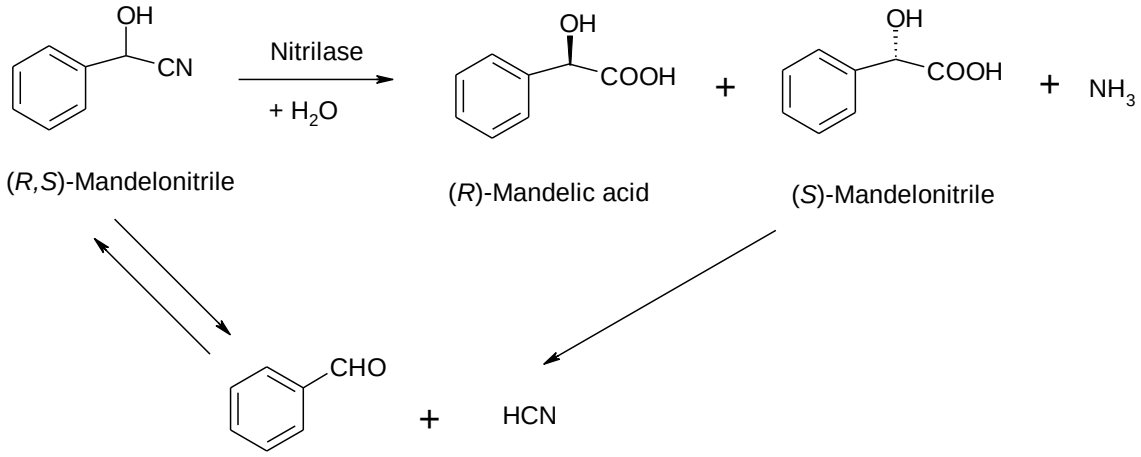


Figure 1.4: Industrial production of (*R*)-mandelic acid (Adapted from Gong *et al.*, 2012)

Nitrilase from *Alcaligenes* sp. was adopted in (*R*)-mandelic acid production on laboratory scale. The biotransformations proceeded in batch and fed batch reactions run in buffer or buffer/organic co-solvent setup (Zhang *et al.*, 2010; Zhang et al, 2011). Immobilized and permeabilized preparations of the biocatalyst were also employed in the reactions (Xue *et al.*, 2013; He *et al.*, 2010; Zhang *et al.*, 2015). Lately, total mass of 450 g of optically pure (*R*)-mandelic acid were produced from a fed-batch reaction (16 feeds of 100 mM substrate) by glutaraldehyde cross-linked recombinant *E. coli* cells expressing the nitrilase gene from *Alcaligenes* sp. (Zhang *et al.*, 2014). Further

improvement of the biocatalyst efficiency was made by isopropanol permeabilization of the *E. coli* cells (Zhang *et al.*, 2015).

The enzymatic production of (*S*)-mandelic acid may prove problematic as no nitrilase with a satisfactory (*S*)-selectivity has been identified so far (Rantwijk and Stolz, 2015). A solution was proposed in the form of a bi-enzymatic process. It starts with the synthesis of the enantiopure (*S*)-mandelonitrile from aldehyde and HCN by hydroxynitrile lyase. The following step is the hydrolysis of the (*S*)-mandelonitrile by nitrilase with a low enantioselectivity (Rustler *et al.*, 2008; Sosedov *et al.*, 2009). The bienzymatic cascade can be carried out in "one pot" by co-expressing both enzymes in one host. The gene of (*S*)-hydroxynitrile lyase from cassava (*Manihot esculenta*) together with the gene of arylacetonitrilase from *Pseudomonas fluorescens* EBC191 were expressed in *E. coli* (Sosedov *et al.*, 2009) or in *Pichia pastoris* (Rustler *et al.*, 2008). Recently, a combi-CLEA of the two enzymes plus an amidase from *Rhodococcus erythopolis* afforded (*S*)-mandelic acid as the sole product in 90% yield and >99% enantiomeric purity (Fig. 1.5).

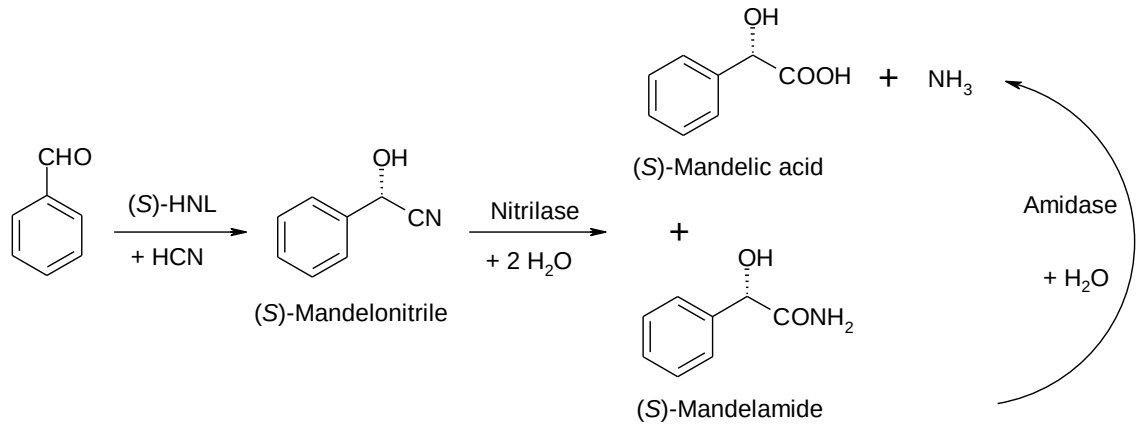


Figure 1.5: Combi-CLEA tri-enzymatic production of (*S*)-mandelic acid (Adapted from Chmura *et al.*, 2013). (S)-HNL…hydroxynitrile lyase

(*R*)-*o*-Chloromandelic acid serves as the precursor of a cardiovascular drug Clopidogrel® (Osprian *et al.*, 2003; Ema *et al.*, 2008). This mandelic acid derivative can

be also produced by the hydrolysis of the parent nitrile using nitrilases. Bi-phasic systems were adopted in these bioconversions. Nitrilase from *Labrenzia aggregata* converted 300 mM (*R*)-*o*-chloromandelonitrile in 10 % (v/v) toluene as a co-solvent and the product e.e. reached 96.5 % (Zhang *et al.*, 2012). Later, (*R*)-*o*-Chloromandelic acid was produced in 415 mM concentration and 97.6% e.e. by a nitrilase from *Burkholderia cenocepacia* J2315 (Wang *et al.*, 2014). The experiment was carried out in a fed-batch mode using ethanol as a cosolvent (20 % v/v).

1.3.3 Acrylic acid

Acrylic acid (2-propenoic acid) is a commodity chemical with an estimated annual production capacity of 4.2 million metric tons. Major uses of acrylic acid, its salt and esters, are in the production of polymeric flocculants, dispersants, coatings, paints, adhesives, and binders for leather, paper and textile (Straathof *et al.*, 2005). Currently, most commercial acrylic acid is produced by partial oxidation of propene, a process which generates several unwanted by-products such as acrolein and a large amount of inorganic waste (Shen *et al.*, 2009). The enzymatic hydrolysis of acrylonitrile using nitrilase is currently considered an attractive alternative for the synthesis of acrylic acid (Gong *et al.*, 2012).

The nitrilase producing *R. rhodochrous* J1 afforded 390 g L^{-1} acrylic acid (Nagasawa *et al.*, 1990b). A mutant strain of *R. rhodochrous* tg1-A6 with high nitrilase activity afforded 414.5 g L^{-1} acrylic acid, which was accumulated by transforming 43 feeds of acrylonitrile during a 10-h reaction (Luo *et al.*, 2006).

1.3.4 Glycolic acid

Glycolic acid, the simplest α-hydroxycarboxylic acid, is used in skin care products, industrial cleaners and as a monomer in the synthesis of polyglycolic acid for dissolvable sutures, drug-delivery and packaging materials. Majority of the commercially available glycolic acid is currently produced through high-pressure and

high-temperature reaction of formaldehyde and carbon monoxide under acid catalysis (Martínková and Křen, 2010; Gong *et al.*, 2012).

Several studies appeared recently, proposing enzymatic catalysis for the glycolic acid manufacture. Immobilized *E. coli* cells producing the *Acidovorax facilis* 72W nitrilase mutant were adopted in a chemoenzymatic process (Fig. 1.6), and a biocatalyst productivity of >1,000 g glycolic acid g_{dcw}^{-1} was achieved (Panova *et al.*, 2007). A productivity of 1,010 g glycolic acid g^{-1} cells after 55 recycle reactions was achieved with a mutant *A. facilis* nitrilase (Wu *et al.*, 2008).

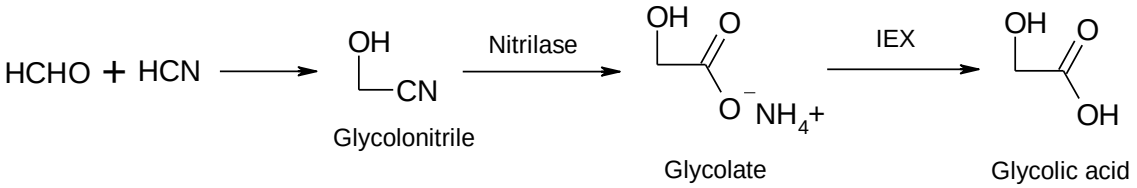


Figure 1.6.: Chemoenzymatic process using mutant nitrilase from *Acidovorax facilis* 72W for the synthesis of glycolic acid from formaldehyde and hydrogen cyanide (Adapted from Panova *et al.*, 2007). IEX…ion exchange

1.3.5 Biodegradation

Nitrile compounds used in chemical industry and agriculture are relevant pollutants with different degrees of toxicity (Saillenfait and Sabaté, 2000; Mylerová and Martínková, 2003) and have been detected in samples from diverse environments, such as sewage sludge from a water-treatment plant and samples collected from marine and coastal areas (Fang *et al.*, 2015). The typical contaminanting compounds include acetonitrile, acrylonitrile, succinonitrile, benzonitrile, and the herbicides dichlobenil, bromoxynil, and ioxynil. Bioremediation with microorganisms harboring nitrile-converting enzymes offers an efficient method for removing these contaminants (Gong *et al.*, 2012).

Bacterial strains of the genus *Rhodococcus* proved to be a promising option for the clean-up of polluted sites, because of their large genomes and their wealth of

catabolic pathways which enable them to uptake and metabolize various hydrophobic compounds (Martínková *et al.*, 2009).

Rhodococcus rhodochrous BX2, an isolate from the contaminated soil, was reported to use acetonitrile, acrylonitrile and crotononitrile as the sole carbon and nitrogen sources, resulting in the complete degradation of these compounds. Both NHase/amidase and nitrilase enzyme systems were involved in the nitrile consumption (Fang *et al.*, 2015).

The soil isolates *Rhodococcus rhodochrous* PA-34, *Rhodococcus* sp. NDB 1165 and *Nocardia globerula* NHB-2 grown on isobutyronitrile as inducer expressed nitrilases with activities towards benzonitrile. In the form of resting cell suspensions, the strains were used for degradation of 0.5 mM benzonitrile herbicides chloroxynil, bromoxynil and ioxynil. Depending on the strain and compound, 30 to 100 % of the herbicides were hydrolyzed after 5-h incubation. An almost full conversion of chloroxynil and bromoxynil and ca. 60 % conversion of ioxynil was observed in all strains within 20-h icubation. The resulting metabolites in the form of the corresponding carboxylic acids were shown to be less toxic than the parent compounds (Veselá *et al.*, 2010).

2 Aims of the study

- to select the sequences of new nitrilases in genomes of filamentous fungi and bacteria

- to express the genes of hypothetical nitrilases in *Escherichia coli* and to purify them

- to characterize the purified enzymes in terms of substrate specificity, kinetics and size of subunit and holoenzyme

- to prepare samples for structural analyses (analytical ultracentrifugation, electron microscopy) and cooperate on structural analysis and homologous modeling

- to apply the new nitrilases in the hydrolysis of substrates with industrial impact such as (*R,S*)-mandelonitrile and precursors of the taxol sidechain; to optimize the process of (*R*)-mandelic acid production on laboratory scale

3 Results and Discussion

3.1 Expression of fungal nitrilase genes in *E. coli*

Nitrilases from archaea, bacteria and plants have been previously produced in heterologous hosts (for reviews see Gong *et al.*, 2013; Gong *et al.*, 2012; Piotrowski 2008). Recently, expression of a number of fungal nitrilase genes in *E. coli* was performed by our research group (Kaplan *et al.*, 2011; Petříčková *et al.*, 2012a).

The genes were obtained by mining the databases for sequences of putative nitrilases similar to the previously produced, partially purified and characterized fungal nitrilases.

In contrast to the aromatic nitrilases (Kaplan *et al.*, 2006c; Vejvoda *et al.*, 2008), expression of fungal arylacetonitrilase genes in the native organisms poses several drawbacks such as specific conditions of growth and induction. Rapid activity loss after harvest and tissue disruption makes the enzyme purification difficult or impossible (Petříčková, 2013). In constrast, the expression of the corresponding genes in *E. coli* eliminates these problems, and in combination with genome mining, it is a suitable way to test a number of new enzymes.

The gene and protein databases have become a rich source of nitrilase sequences, and the subsequent gene synthesis – including the option of codon frequency optimization – and expression in *E. coli* has become a convenient method for nitrilase production and purification. Furthermore, with the previously obtained results in mind, the search for new nitrilases can be focused on expected properties. Comparing not only the overall sequence similarities, but also looking for the presence of specific regions may prove useful for the selection of enzymes with desired substrate specificities (Seffernick *et al.*, 2009).

In the present work, new putative nitrilase sequences were selected according to their similarity to the previously studied enzymes. Together with our previously characterized nitrilases (Petříčková *et al.*, 2012a), they were produced in *E. coli* BL21 (DE3) Gold, and tested, in the form of whole-cell catalyst, against a set of different

substrates, consisting of aromatic, arylaliphatic and aliphatic nitriles (for more details see Appendix 1).

In the group of 12 heterologously produced fungal enzymes, we confirmed 3 aromatic nitrilases, 6 arylaliphatic nitrilases, 1 nitrilase with a mixed substrate preference and 2 cyanide hydratases. The results showed us that there were correlations between the amino acid sequences of the enzymes and their substrate specificities. A phylogenetic tree of all nitrilases heterologously produced by us to date, including bacterial and plant-like enzymes, is divided into five branches (Fig. 3.1; some of these enzymes are not discussed in this work). Within these branches, the presence of specific motifs in the enzyme sequences can be recognized near the active cysteine residue (Fig. 3.2; for complete sequence alignments see Supplementary Fig. S1-S3). With two exceptions, enzymes clustered in these branches also share the same substrate preference.

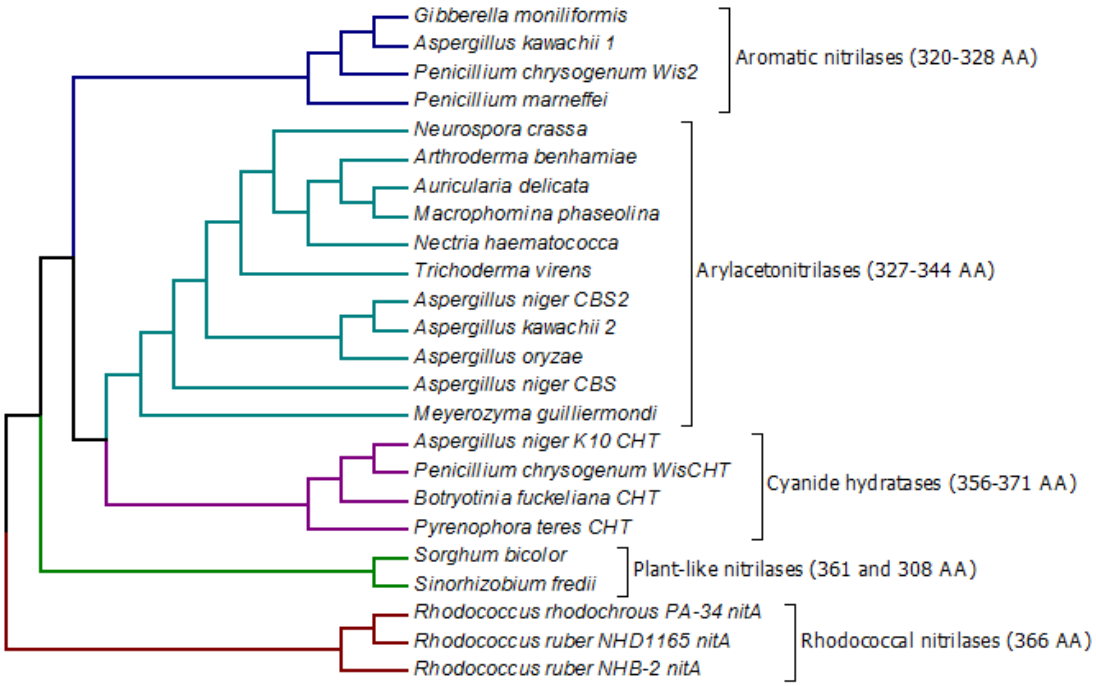


Figure 3.1: Phylogenetic tree of characterized fungal nitrilases and cyanide hydratases and plant-like and rhodococcal nitrilases

```
Aromatic and                    NitGm   FG------RVAGLNCWEHTQTLLR
related nitrilases              NitMg   FK-EAGPVEVGCLSCWEHMQPLLY
                                NitPc2  FG------RIGGLNCWEHTQPLLR
                                NitPm   FG------KVGGLNCWEHLQPLLR
                                NitRr1  FA------RLGALNCWEHFQTLTK
                                        *        :: * **** * *

Arylacetonitrilases             NitAb   FGGDIGVVKVGTLACWEHALPLLK
                                NitAn   IG------KVGALACWEHIQPLLK
                                NitAn2  AG------RVGALSCWEHIQPLLK
                                NitAo   VG------RVGALSCWEHIQPLLK
                                NitNc   FGSELGSIKVGTLNCWEHAQPLLK
                                NitNh   FGAEHGKIKVGCFACWEHTQPLLK
                                         *       **    ****  ****

Cyanide hydratases              NitAn1  IG------RVGHLNCWENMNPFMK
                                NitPc1  IG------RIGHLNCWENMNPFLK
                                        **      *:************ *
```

Figure 3.2: Sequence motifs near the active cysteine residue in fungal aromatic and arylaliphatic nitrilases and cyanide hydratases

Fig. 3.3 shows the preferred aromatic substrates of the nitrilases from *Gibberella moniliformis* (NitGm), *Penicillium marneffei* (NitPm), *Penicillium chrysogenum* (NitPc) and *Meyerozyma guiliermondii* (NitMg).

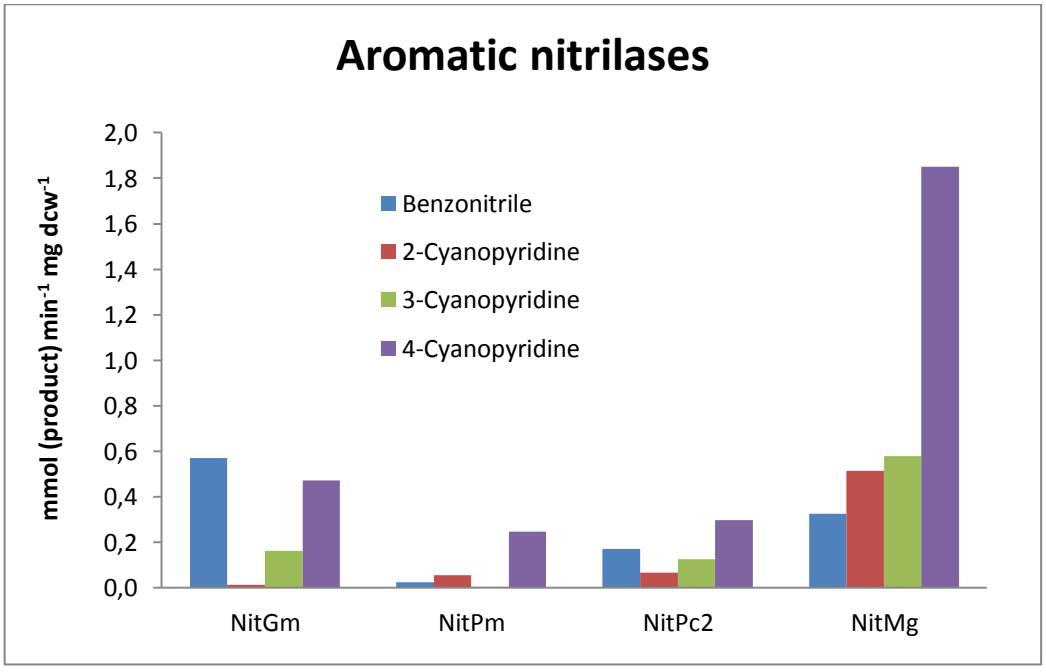


Figure 3.3: Activities of *E. coli* cells producing codon-optimized fungal aromatic nitrilases

The aromatic nitrilases from *Gibberella moniliformis* (NitGm), *Penicillium marneffei* (NitPm) and *Penicillium chrysogenum* (NitPc2) shared 43-56 % sequence identities. They preferred benzonitrile and/or 4-cyanopyridine as their substrates (Fig 3.3) and generally displayed a lower activity than arylacetonitrilases (Fig. 3.4; see below). The nitrilase with a mixed substrate preference, i.e. the enzyme from *Meyerozyma guiliermondii* (NitMg) exhibited a different substrate specificity pattern (Fig. 3.3; see below). Aromatic nitrilases were previously studied in bacteria (*Bacillus pallidus* Dac521, *Nocardia* sp. NCIB 11216, *Rhodococcus rhodochrous* NCIMB 11216, *Pseudomonas aeruginosa* 10145, *Geobacillus pallidus* RAPc8), and fungi (*Fusarium solani* O1, *Aspergillus niger* K10 and *Fusarium oxysporum* f. sp. *melonis*; see Gong *et al.*, 2012 for a review). Nitrilases with activities for (hetero)aromatic nitriles can be employed in the production of nicotinic and isonicotinic acid from 3- and 4-cyanopyridine, and several studies using fed-batch or continuous flow set-ups with bacterial nitrilase (Mathew *et al.*, 1988; Almatawah and Cowan, 1999; Sharma *et al.*, 2006; Prasad *et al.*, 2007) or fungal nitrilase (Vejvoda *et al.*, 2006a; Vejvoda *et al.*, 2006b; Malandra *et al.*, 2009) have been published. Suitable immobilization methods may improve the enzyme's stability and reusability, as in the case of the nitrilase-harboring cells of the fungus *Gibberella intermedia* CA3-1, in which a production of up to 205.7 g g_{DCW}^{-1} of nicotinic acid was reported (Li *et al.*, 2015).

The preferred substrates of all arylacetonitrilases, i.e. the enzymes from *Neurospora crassa* (NitNc), *Aspergillus niger* CBS (NitAn2 and NitAn3), *Aspergillus oryzae* (NitAo), *Nectria haematococca* (NitNh) and *Arthroderma benhamiae* (NitAb) were phenylacetonitrile, (*R,S*)-mandelonitrile and indole-3-acetonitrile (Fig. 3.4).

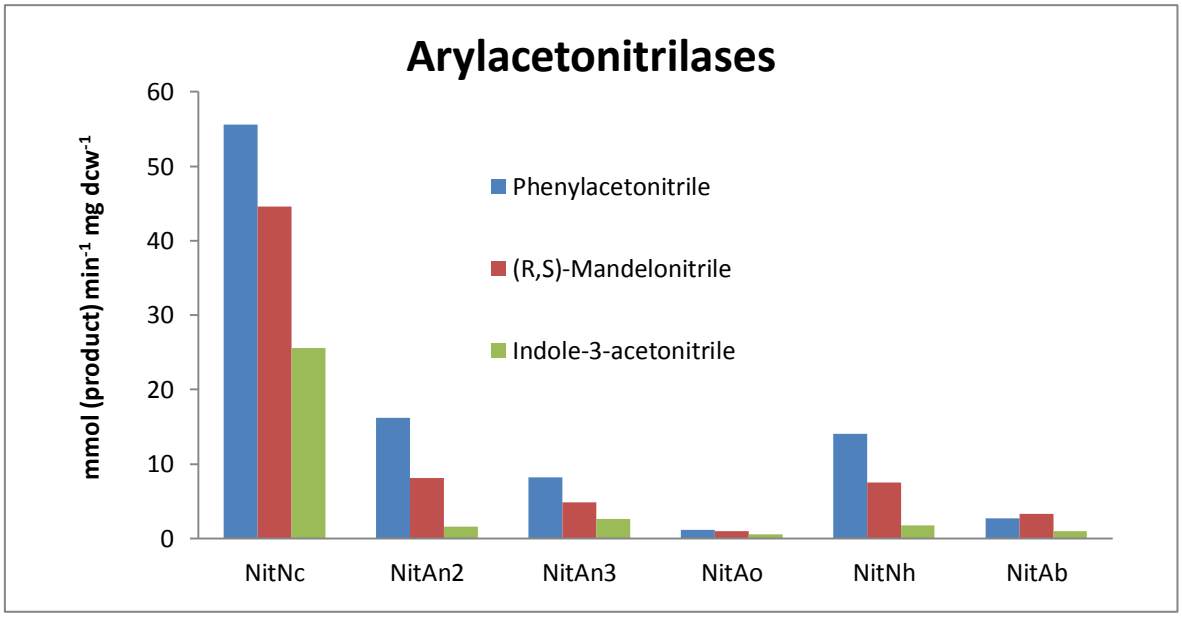


Figure 3.4: Activities of *E. coli* cells producing codon-optimized fungal arylacetonitrilases

The fungal arylacetonitrilases exhibited sequence identities between 49-74 % but shared only about 40 % identities to the known bacterial arylacetonitrilases, such as those from *Alcaligenes faecalis* (Nagasawa *et al.*, 1990a; Yamamoto *et al.*, 1992), *Alcaligenes* sp. (Zhang *et al.*, 2011) and *Pseudomonas putida* (Banerjee *et al.*, 2009). Like the bacterial arylacetonitrilases, their fungal counterparts differed between each other in their enantioselectivity for (*R*)-mandelonitrile (NitNc and NitAn2 >99 % e.e., NitAn3, NitAo, NItNh and NitAb 63-89 % e.e.) and the percentage of mandelamide in the reaction product. These specific features are yet impossible to predict from the nitrilase sequences, however, some studies on various nitrilase mutants revealed the effect of certain amino acid residues on enantioselectivity and amide production (Kiziak *et al.*, 2007; Kiziak and Stolz, 2009; Sosedov *et al.*, 2010; Petříčková *et al.*, 2012b).

The specific activities of NitMg are displayed together with aromatic nitrilases in Fig. 3.3 because of its high activities for (hetero)aromatic nitriles; however, it seems that its substrate specificity is of a mixed type. NitMg had a significantly higher activity for 4-cyanopyridine and 3- and 2-cyanopyridine than the other tested aromatic nitrilases and, moreover, its activity for phenylacetonitrile (0.44 U mg dcw^{-1}), the typical substrate of arylacetonitrilases, was nearly the same as for benzonitrile. On the other

hand, its activity for mandelonitrile was negligible. In the phylogenetic tree (Fig. 3.1), NitMg is clustered within the arylacetonitrilase branch, and furthermore, its sequence contains a hexapeptide motif which is a characteristic feature of many fungal arylacetonitrilases (although not all of these enzymes contain it), and is positioned close to the catalytic cysteine residue (e.g. in NitAb, NitNc, NitNh; Fig. 3.2). Thus, NitMg seems to be an exception from the sequence/substrate specificity correlation of the fungal nitrilases. Its sequence also shared only moderate identities to the sequences of the other studied fungal nitrilases (37-44 %).

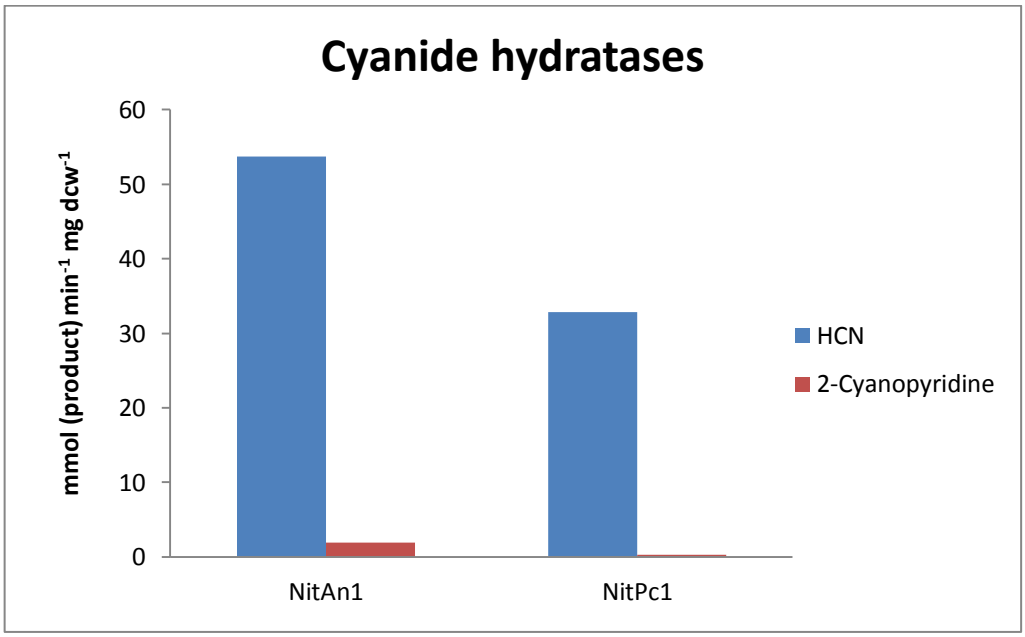


Figure 3.5: Activities of *E. coli* cells producing codon-optimized fungal cyanide hydratases

The enzymes from *Aspergillus niger* K10 (NitAn1) and *Penicillium chrysogenum* (NitPc1) were identified as cyanide hydratases (CHTs). Sequences of these enzymes shared 90 % identity, and on average, they were 28 amino acids longer than the rest of the fungal nitrilases. Both CHTs displayed an almost exclusive preference for HCN as a substrate; however, minor activities for 2-cyanopyridine were observed as well (Fig. 3.5). Activities for saturated and unsaturated aliphatic nitriles or benzonitrile had been previously reported in CHTs from *Fusarium lateritium* (Nolan *et al.*, 2003) and *Fusarium oxysporum* (Yanase *et al.*, 2000), but were only about 0.01-0.04 % of the activity for

HCN. In contrast, the activity for 2-cyanopyridine in NitAn1 was an order of magnitude higher, and represented 3.6 % of that for HCN.

As for general features common for all the tested nitrilases, the catalytic triade Glu-Lys-Cys was found in all studied enzymes. Tryptophan and glutamic acid residues were conserved among all our nitrilases at the positions 1 and 2 downstream of the catalytic cysteine (for examples see Fig. 3.2). An amino acid residue other than tryptophan is very rare at this position. So far, only in bacterial nitrilases from *Klebsiella ozaenae* (O'Reilly and Turner, 2003) and *Pseudomonas fluorescens* (Kiziak *et al.*, 2005), alanine was found instead of tryptophan. The residue at position 3 downstream of the active cysteine is typically a histidine for nitrilases and asparagine for CHTs, although nitrilases with asparagine at this position were reported as well (*K. ozaenae* and *Arabidopsis thaliana*; O'Reilly and Turner, 2003).

3.2 Purification and characterization of recombinant nitrilases from *Arthroderma benhamiae* and *Nectria haematococca*

The synthetic genes coding for arylacetonitrilases from *Arthroderma benhamiae* (NitAb) and *Nectria haematococca* (NitNh) were prepared by GeneArt (Regensburg, Germany; commercial service), ligated into pET30a(+) vector and expressed in *E. coli* Bl21 DE3 Gold (Appendix 2). The cell cultures were inoculated from the cryoconserve and grown in 100 mL of LB-medium in shaken 500-mL Erlenmeyer flasks at 37°C. After the culture OD_{610} reached a value of approx. 0.6, 0.5 mM IPTG was added for protein expression induction, and the incubation temperature was lowered to 25°C. The cultures were harvested by centrifugation after the next 17 hours.

For the nitrilase purification, the cells were resuspended in Tris/HCl buffer with 150 mM NaCl, pH 8.0 and sonicated. After centrifugation, the cell-free extract was applied on ion exchange column, the active fractions were collected and applied on gel filtration column. The active fractions were pooled and concentrated.

Tables 3.1 and 3.2 summarize the enzyme activity and yield after each purification step. In both cases, the nitrilase formed the major part of the soluble

proteins in the cell (Fig. 3.6), therefore NitAb and NitNh were only purified 1.7 and 1.8 fold, respectively. The yields of both enzymes were similar, however, NitNh had nearly 4 times higher specific activity than NitAb.

Table 3.1: Purification of the nitrilase from *Arthroderma benhamiae* (NitAb)

	Specific activity ($U\ mg^{-1}$)	**Total activity (U)**	**Yield (%)**
Cell-free extract	9.4	416	100.0
Ion exchange chromatography	14.7	347	83.3
Gel filtration	16.3	117	28.2

Table 3.2: Purification of the nitrilase from *Nectria haematococca* (NitNh)

	Specific activity ($U\ mg^{-1}$)	**Total activity (U)**	**Yield (%)**
Cell-free extract	36.2	5192	100.0
Ion exchange chromatography	43.5	3835	73.9
Gel filtration	64.4	992	19.1

Fig. 3.6 shows the SDS-PAGE analysis of NitAb and NitNh after each purification step. Appearance of an intense band at ca. 37 and 36 kDa could be observed after induction with IPTG. These apparent molecular weights of the subunits are similar to the theoretical ones (36.1 and 36.8 kDa, respectively), although with slight differences. It is evident from Fig. 3.6 that NitAb appears to have a higher molecular weight than NitNh, despite the fact that the theoretical value is lower. This might be caused by an incomplete denaturation of the protein.

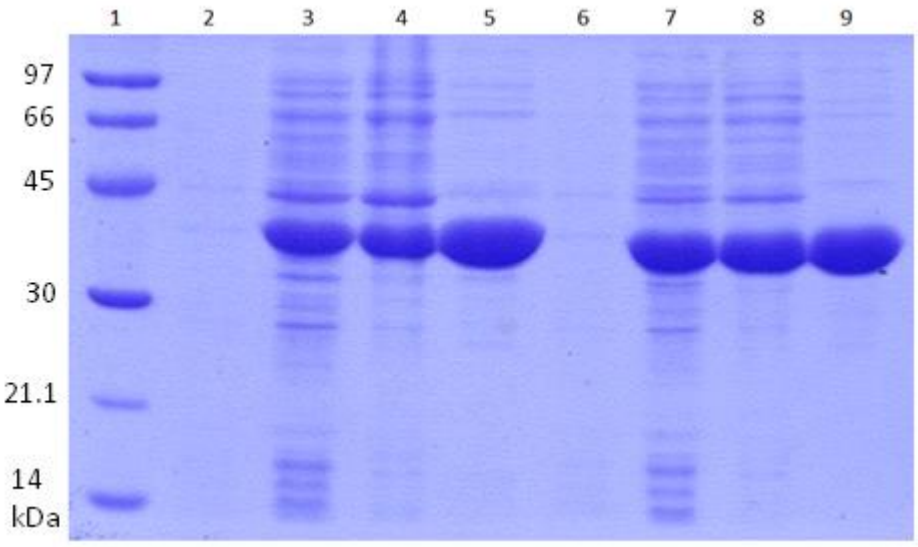


Figure 3.6: SDS-PAGE analysis of nitrilases NitAb (lanes 2-5) and NitNh (lanes 6-9) after each purification step. Lane 1 – marker, lanes 2 and 6 – non-induced cells, lanes 3 and 7 – cell-free extracts, lanes 4 and 8 – ion exchange chromatography, lanes 5 and 9 – gel filtration.

Fig. 3.7 demonstrates the results of SDS-PAGE performed without dithiotreitol in the sample preparation. Without this reducing agent, the disulfide bridges in the proteins should remain intact. The non-reducing SDS-PAGE analysis of the purified NitAb and NitNh revealed a number of other bands of higher molecular mass compared to the monomer. The next most prominent band can be found at approx. 70 kDa, which roughly corresponds to a dimer. It is possible that disulfide bonds may exist between the subunits

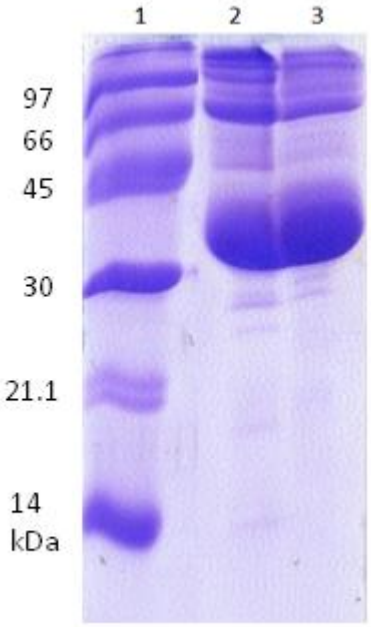


Figure 3.7: Non-reducing SDS-PAGE of the purified nitrilases. Lane 1 – marker, lane 2 – nitrilase NitAb, lane 3 – nitrilase NitNh

Both enzymes exhibited the substrate specificity typical for arylacetonitrilases: their preferred substrates were phenylacetonitrile and (*R,S*)-mandelonitrile. However, their relative activities for these substrates differed. NitAb hydrolyzed the two nitriles with nearly the same activities, whereas NitNh converted mandelonitrile with only 27 % of the activity for phenylacetonitrile (see Tab. 3.3).

As for the kinetic parameters, V_{max} was similar for both substrates in NitAb, but the K_m value for mandelonitrile was nearly 4 times higher. NitNh exhibited almost the same K_m values for both substrates, however, V_{max} for phenylacetonitrile was 3.6 times higher than that for mandelonitrile (see Tab. 3.4). In this aspect, the previously characterized fungal arylacetonitrilase, NitAn2, is similar to NitAb, and another enzyme, NitNc, shows the same trends in its kinetic parameters as NitNh (Petříčková *et al.*, 2012a).

The relative activities of NitAb for other tested substrates were generally higher than those of NitNh (Table 3.3).

The amide production was also different for each enzyme. NitNh generally produced more amide than NitAb. From phenylacetonitrile and mandelonitrile, NitNh

produced more than 6 % amide of the total product. From 3-cyanopyridine it was even 9.9 %. In contrast, NitAb produced only 1.6 % and 0.4 % of amide from mandelonitrile and 3-indolylacetonitrile, respectively; other substrates were exclusively converted to acids. Both enzymes also displayed minor activity for HCN, whose only reaction product was formamide.

Table 3.3: Substrate specificity of nitrilases NitAb and NitNh

Substrate	Relative activity (%) / amide (molar % in total product)	
	NitAb	**NitNh**
Phenylacetonitrile	100[a] / 0	100[a] / 6.5
(*R,S*)-Mandelonitrile	94 / 1.6	27 / 6.2
(*R,S*)-2-Phenylpropionitrile	1.3 / 0	0.14 / 0
3-Indolylacetonitrile	44 / 0.4	15 / 1.6
3-Phenylpropionitrile	5 / 0	0.03 / 0
KCN	2.5 / 100	0.12 / 100
Benzonitrile	0.5 / 0	0.1[b] / 0
3-Chlorobenzonitrile	0.3 / 0	0.1[b] / 0
4-Chlorobenzonitrile	2.3 / 0	0.3[b] / 0
2-Cyanopyridine	8 / 0	1.9[b] / 9.9

3-Cyanopyridine	1 / 0	2.7[b] / 0
4-Cyanopyridine	9 / 0	7.5[b] / 0.6

Activities assayed with 25 mM substrate, except KCN (1 mM)

[a] 16.3 and 64.4 U mg^{-1} protein taken as 100% for the purified nitrilases NitAb and NitNh, respectively

[b] adopted from Veselá, 2011

Table 3.4: Kinetic parameters of nitrilases NitAb and NitNh

	NitAb		NitNh	
	K_m (mM)	V_{max} (U mg^{-1})	K_m (mM)	V_{max} (U mg^{-1})
Phenylacetonitrile	1.4	25.7	8.3	111.1
(*R,S*)-Mandelonitrile	4.0	20.5	9.9	31.3

Both enzymes hydrolyzed 25 mM (*R,S*)-mandelonitrile with selectivity for its (*R*)-enantiomer. NitAb was only moderately selective, whether the reaction pH was 5.0 or 8.0. NitNh displayed a higher e.e. value at pH 5.0 than NitAb, although still in the moderate region. At pH 8.0, however, the e.e. value rose to 89 % (Tab. 3.5).

In contrast, NitAn2 and NitNc were highly (*R*)-selective even at pH 5.0 (e.e. > 90 %), although NitNc produced 40 % of (*S*)-mandelamide under these conditions. At pH 7.0-7.5, NitAn2 and NitNc formed (*R*)-mandelic acid with e.e. over 99 %. NitNc also produced only 15 % of (*S*)-mandelamide and with a lower e.e (51 %). NitAn2 produced no mandelamide both at pH 5.0 or higher (Petříčková *et al.*, 2012b).

The explanation of the higher e.e. values might be the effect of lower substrate concentration (10 mM) in case of NitAn2 and NitNc. Mandelonitrile in a solution undergoes decomposition into benzaldehyde and HCN and there is an equilibrium between these compounds. At lower pH (5.0) the substrate racemization is slow, and therefore the availability of the preferred (*R*)-enantiomer is limited. Higher pH allows a rapid racemization of mandelonitrile, and the (*R*)-enantiomer is available in sufficient

amount during the entire course of the reaction. However, the moderate enantioselectivity of NitAb seems to be a given characteristic of the enzyme that can be only slightly affected by the reaction conditions.

Table 3.5: Hydrolysis of 25 mM (*R,S*)-mandelonitrile to (*R*)-mandelic acid by the nitrilases NitAb and NitNh – effect of pH on enantioselectivity

pH	E.e. (%) (*R*)-mandelic acid	
	NitAb	NitNh
5.0	64	67
8.0	63	89

The (*R*)-selective hydrolysis of (*R,S*)-mandelonitrile seems to be a common property of arylacetonitrilase. To date, bacterial arylacetonitrilases have been quite extensively studied in terms of their enantioselectivity for (*R,S*)-mandelonitrile, and enzymes with excellent, moderate or no (*R*)-selectivity were discovered.

The highly (*R*)-selective arylacetonitrilases were found in the *Alcaligenes* genus (Nagasawa *et al.*, 1990; Yamamoto *et al.*, 1992; Zhang *et al.*, 2010; Liu *et al.* 2011) and were used for the production of high concentrations of (*R*)-mandelic acid (He *et al.*, 2010; Xue *et al.*, 2013; Zhang *et al.*, 2014a; Zhang *et al.*, 2015).

An example of moderately enantioselective bacterial arylacetonitrilase is the enzyme from *Pseudomonas fluorescens* (Kiziak *et al.*, 2005) which was employed in an enantioretentive biotransformation of (*S*)-mandelonitrile into (*S*)-mandelic acid or (*S*)-mandelamide (Rustler *et al.*, 2008; Sosedov *et al.*, 2009; Chmura *et al.*, 2013).

The nonenantioselective arylacetonitrilases were identified in *Bradyrhizobium japonicum* (Seffernick *et al.* 2009; Zhu *et al.* 2007) and *Burkholderia xenovorans* (Seffernick *et al.* 2009).

NitNc, NitAb and NitNh share only 36–41 % identities with the arylacetonitrilases from the *Alcaligenes*, *Pseudomonas*, *Bradyrhizobium* and *Burkholderia* genera; all three enzymes also possess a hexapeptide insert, a sequence near the active cysteine typical for most fungal arylacetonitrilases. NitAn2, on the

contrary, shares more identities with the bacterial enzymes (41-45 %) and lacks the hexapeptide sequence.

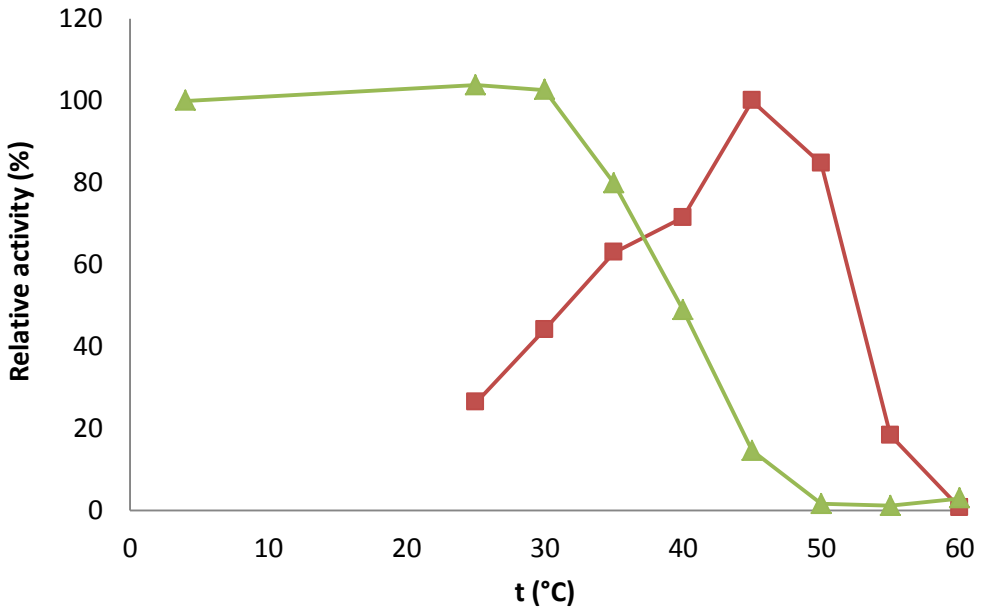


Figure 3.8: Temperature optimum (squares) and stability (triangles) of nitrilase NitAb

Stability of an enzyme is one of the most important criteria for its application in biotechnology. As for the temperature profiles, the optima were 40°C for NitNh (Veselá, 2011) and 45°C for NitAb (Fig. 3.8), however, both enzymes were unstable at temperatures above 30°C (Fig. 3.8).

In contrast, both enzymes were highly active in a broad range of pH: NitNh at pH 5.5-8.5 (Veselá, 2011) and NitAb at 5.0-9.0 (Fig. 3.9). NitNh was stable at pH 5.0-9.0 (Veselá, 2011), NitAb at pH 5.0-10.0 (Fig.3.9), which qualifies them as more pH resistant than a number of bacterial arylacetonitrilases that were stable at pH 6.5-8.0 (see Gong *et al.*, 2012 for a review). Even among the fungal nitrilases the pH stability was usually narrower, the exception being the aromatic nitrilase NitGm, which was stable at pH 6.0-11.0 (Petříčková *et al.*, 2012a).

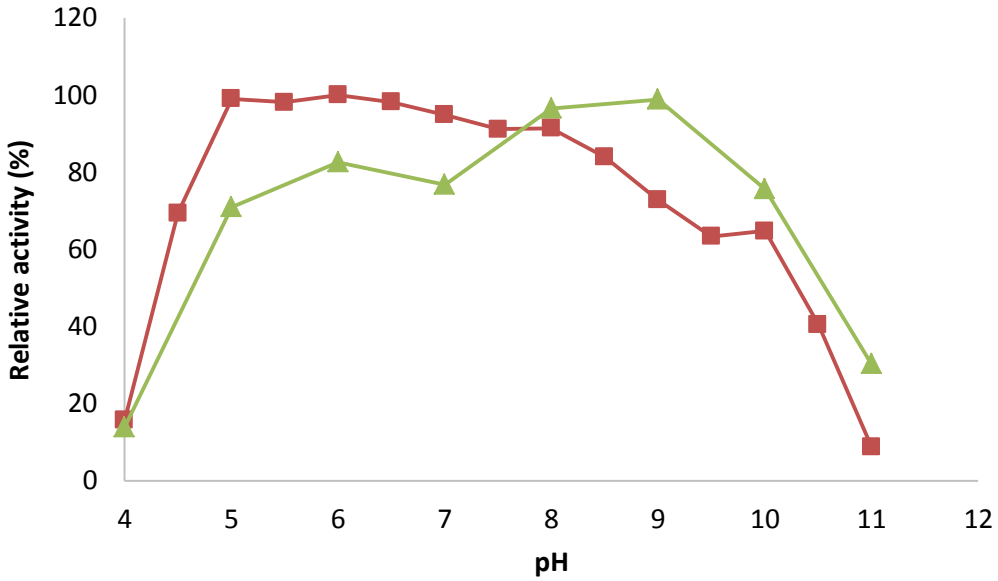


Figure 3.9: pH optimum (squares) and stability (triangles) of nitrilase from NitAb

The purified nitrilases NitAb and NitNh were analyzed by gel filtration (see Table 3.6). The molecular weights of the holoenzymes were 336 and 360 kDa, respectively. This means that the number of subunits in these structures corresponds approximately to ten.

Table 3.6: Molecular weight of the holoenzymes of nitrilases NitAb and NitNh according to gel filtration analysis

Enzyme source	Subunit Mw (kDa)	Holoenzyme Mw (kDa)	Number of subunits
NitAb	36.1	336	9.3
NitNh	36.8	360	9.8

In contrast, the sedimentation velocity analysis revealed the presence of relatively sharp peaks representing dimers, tetramers, hexamers and dodecamers in

the sample of purified NitAb (see Fig. 3.10). These discreet values are rather rare, as the formation of oligomeric rods or helices has been documented for the enzymes of the nitrilase branch of the nitrilase superfamily of enzymes (Thuku *et al.*, 2009; Jandhyala *et al.*, 2003; Vejvoda *et al.*, 2008).

The electron micrographs of NitAb in Fig. 3.12 roughly correspond with these findings, as dimers, tetramers and hexamers can be found in them.

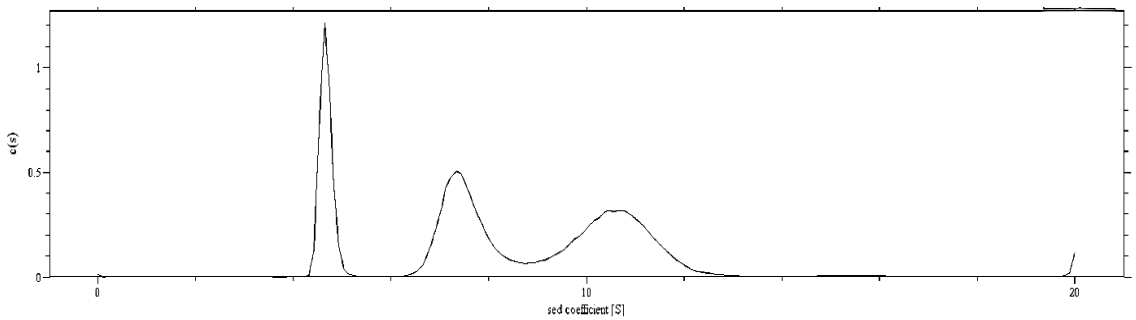


Figure 3.10: Sedimentation velocity analysis of nitrilase NitAb. From the left to the right, the peaks represent a dimer, a tetramer, a hexamer and a trace of dodekamer. (Author: RNDr. Ondřej Vaněk, Ph.D., Department of Biochemistry, Faculty of Science, Charles University in Prague)

In NitNh, the peaks are more difficult to distinguish. Nevertheless, a dimer, a tetramer and a hexamer could be recognized, and further a protein species corresponding to an assembly of 12-24 subunits (Fig. 3.11). The electron micrograph of NitNh (Fig. 3.13) reveals the presence of both short and long oligomers of this enzyme.

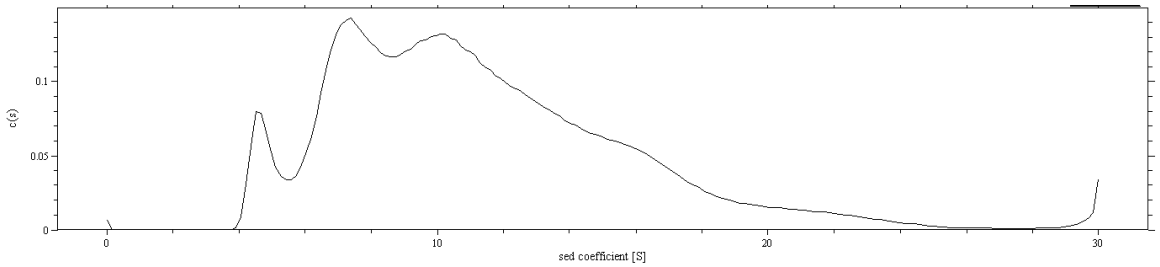
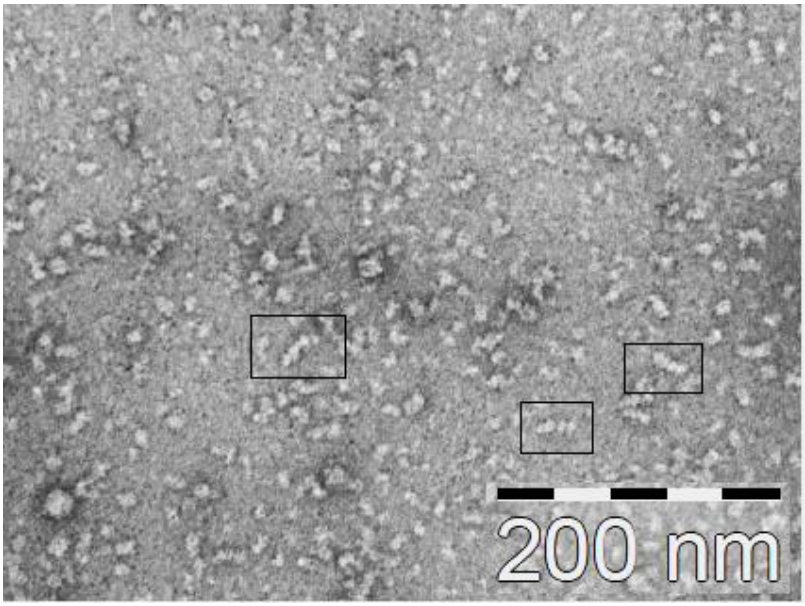


Figure 3.11: Sedimentation velocity analysis of nitrilase NitNh. From the left to the right, the peaks represent a dimer, a tetramer and a hexamer. The following tail represents oligomeric structures of 12-24 subunits. (Author: RNDr. Ondřej Vaněk, Ph.D., Department of Biochemistry, Faculty of Science, Charles University in Prague)

A



B

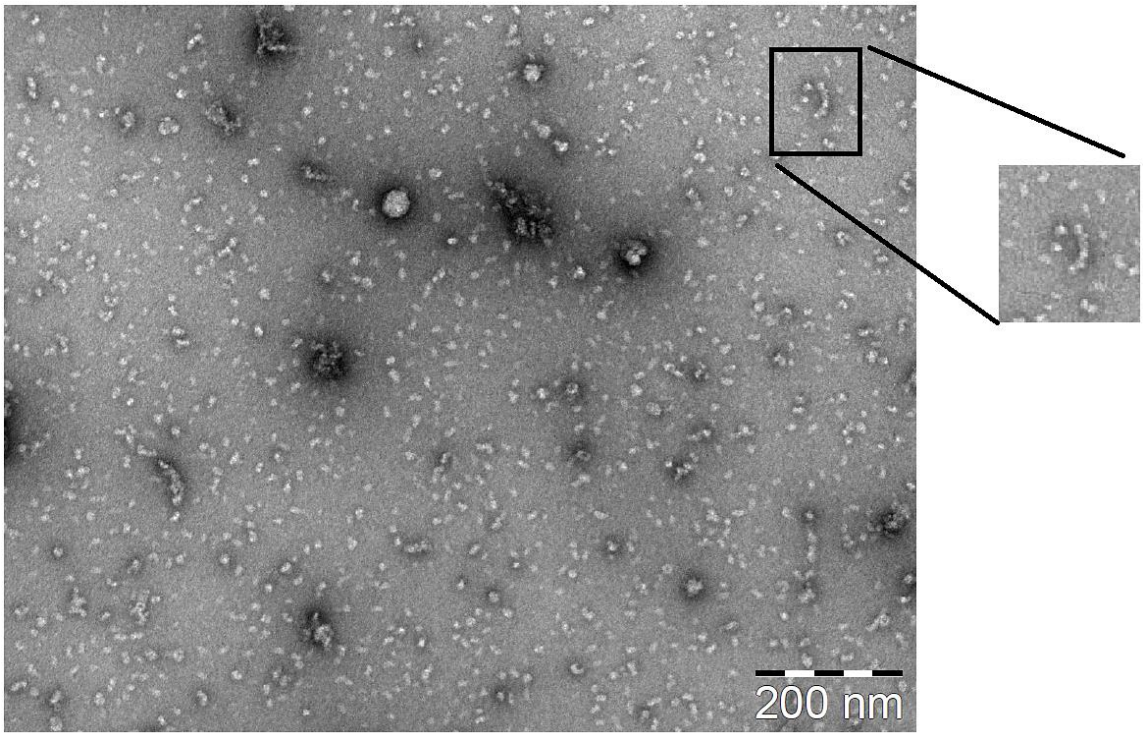


Figure 3.12: Electron micrographs of the nitrilase NitAb with highlighted tetramers (A) and hexamer (B). (Author: RNDr. Oldřich Benada, Ph.D., Institute of Microbiology, Academy of Sciences of the Czech Republic)

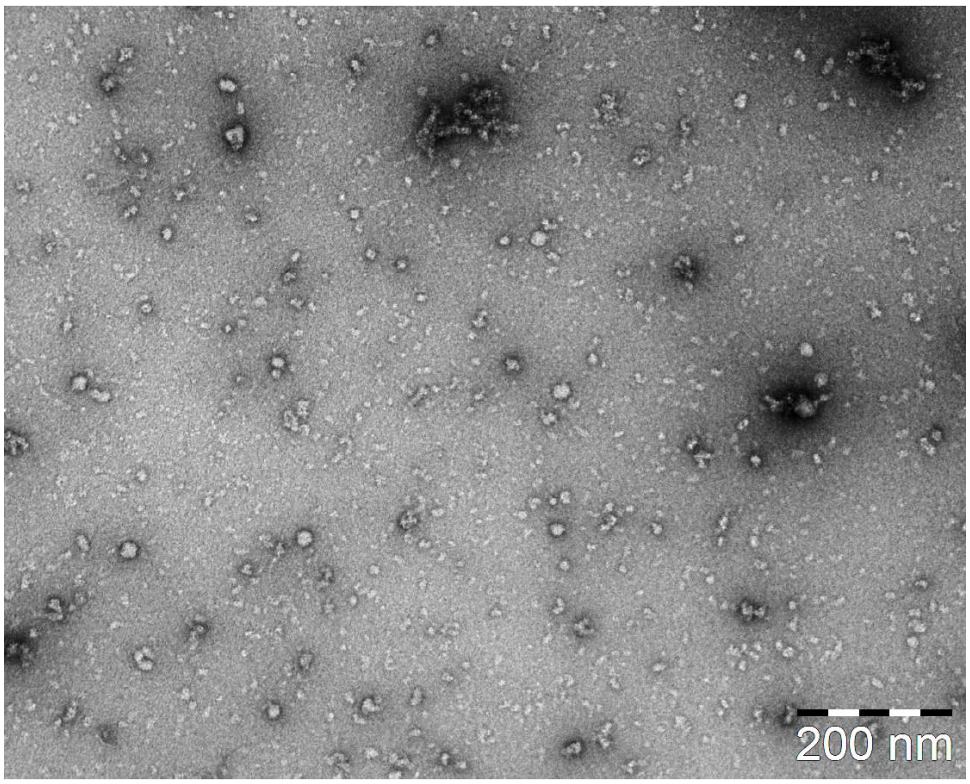


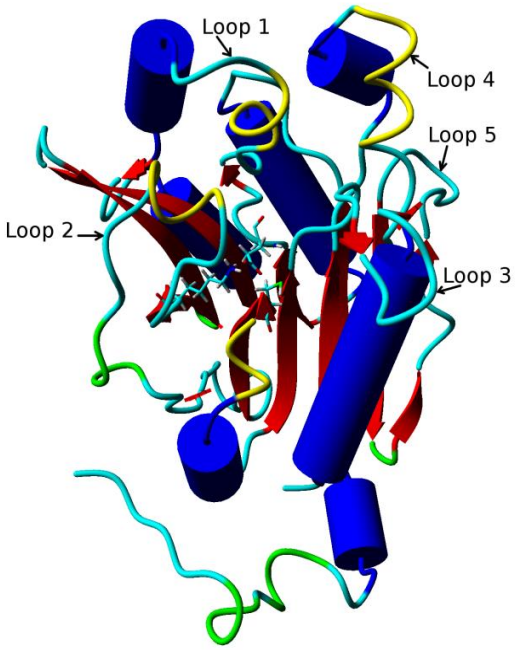
Figure 3.13: Electron micrograph of the nitrilase NitNh (Author: RNDr. Oldřich Benada, Ph.D., Institute of Microbiology, Academy of Sciences of the Czech Republic)

The homology models of NitAb and NitNh were created at the Department of Structure and Function of Proteins, Institute of Nanobiology and Structural Biology of GCRC ASCR by Dr. Natallia Kulik, PhD.

Three proteins of the nitrilase superfamily were used as templates for generating homology models of NitAb and NitNh: a nitrilase from the cyanobacterium *Syechocystis* sp. (Zhang *et al.*, 2014b; Pdb code: 3wuy), a nitrilase from the archaebacterium *Pyrococcus horikoshii* (Sakai *et al.*, 2004; Pdb code: 1j31) and mouse nitrilase (Barglow *et al.*, 2008; Pdb code: 2w1v). The best template was the *Synechocystis* sp. nitrilase, as the identities were between 30-33 % and the coverage 92-93 %. The template search was done by BLAST program (Altschul *et al.*, 1997) and the multiple sequence alignment (see Supplementary Fig. S4) constructed by T-Coffee server (Notredame *et al.*, 2000).

In the homology models of NitAb (Fig. 3.14) and NitNh (Fig 3.15,) the typical nitrilase α-β-β-α sandwich fold of the enzyme subunit can be recognized.

A



B

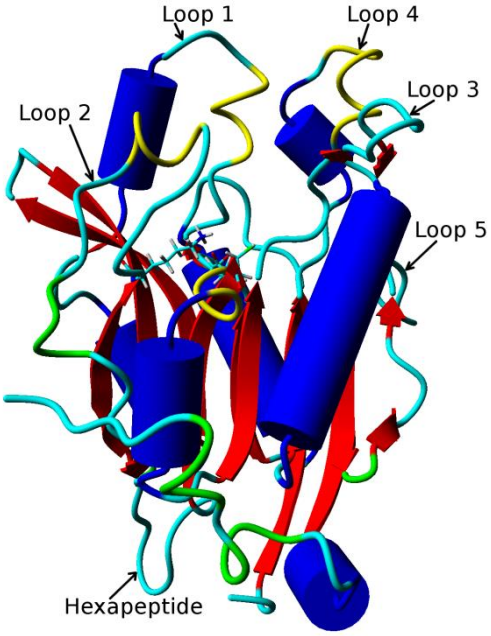
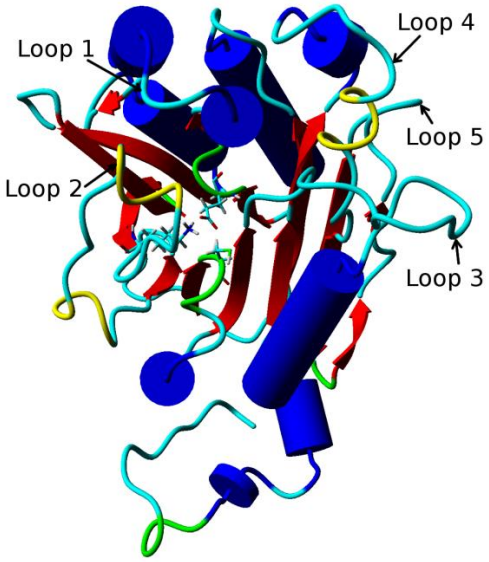


Figure 3.14: Homology models of the nitrilase NitAb - (A) top view, (B) side view. (Author: Dr. Natallia Kulik, PhD., Department of Structure and Function of Proteins, Institute of Nanobiology and Structural Biology of GCRC ASCR)

A



B

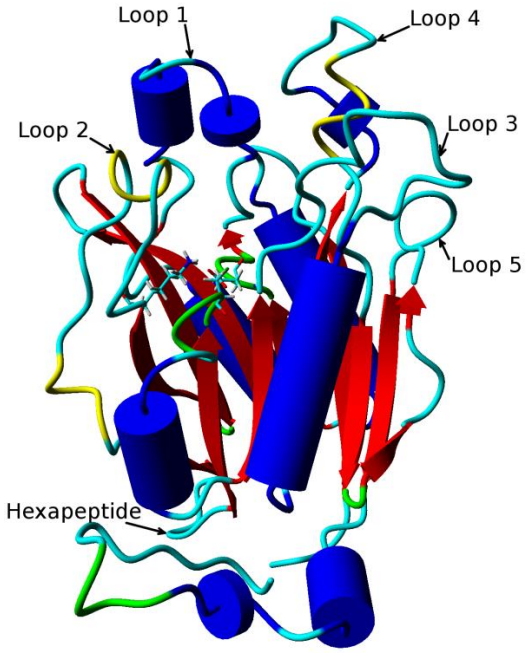


Figure 3.15: Homology models of the nitrilase from NitNh - (A) top view, (B) side view. (Author: Dr. Natallia Kulik, PhD., Department of Structure and Function of Proteins, Institute of Nanobiology and Structural Biology of GCRC ASCR)

Two loops are longer in the fungal nitrilases than in the templates –loop 2 and loop 4. In addition, a hexapeptide, which is a typical feature of fungal arylacetonitrilases, is missing in the templates. Loop 2 is close to the active site and covers the entrance, whereas the hexapeptide is positioned relatively far from the active site. This short sequence is present in both NitAb and NitNh and also in NitNc, that shares 66 and 67 % amino acid identities with NitAb and NitNh.

NitNc has the hexapeptide at positions 155-160, that is in the vicinity of the catalytic cysteine residue. Similar inserts of the same length were found in almost all hypothetical fungal nitrilases homologous with NitNc. However, the homology model of NitNc also revealed that its hexapeptide loop is relatively distant from the active site (Petříčková, 2013) and therefore the probability of its interference with the catalytically active residues is low. It may be possible though, that within the oligomers, the hexapeptide of one subunit interacts with regions near the active site of another subunit.

3.3 Recombinant fungal nitrilases in hydrolysis of high concentrations of (*R,S*)-mandelonitrile

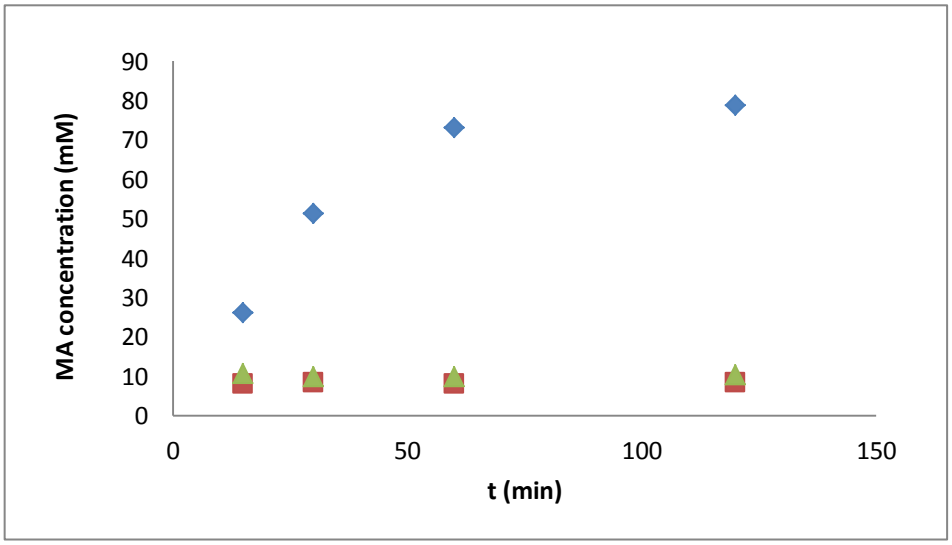
Nitrilases NitAb, NitNh, NitAn2 and NitNc were employed in the production of high concentrations of (*R*)-mandelic acid from (*R,S*)-mandelonitrile (Appendix 3). The experiments were performed in various volumes, from 10 ml in falcon tubes, to 100 ml in Erlemeyer flasks. Concentrations of the reaction substrate, (*R,S*)-mandelonitrile, ranged from 100 – 500 mM, and the reactions were performed at different pH values and with various portions of toluene in the reaction mixture (see below). All the experiments were carried out at 30°C except where stated otherwise.

The initial experiments were performed in 10 ml of the reaction mixture, comparing the reaction rates at 100, 250 or 500 mM (*R,S*)-mandelonitrile concentration at pH 5.0 or 8.0.

At pH 5.0, NitAb (Fig. 3.16) hydrolyzed only 100 mM mandelonitrile at a significant rate, 0.25 and 0.5 M substrate concentrations seemed to be detrimental for the enzyme. At pH 8.0, however, the conditions were more suitable for the enzyme to hydrolyze even 0.5 M mandelonitrile, and almost 90% conversion was achieved within 24 hours.

For NitNh, both low pH and concentrations of mandelonitrile above 250 mM were unsuitable (Fig. 3.17).

A



B

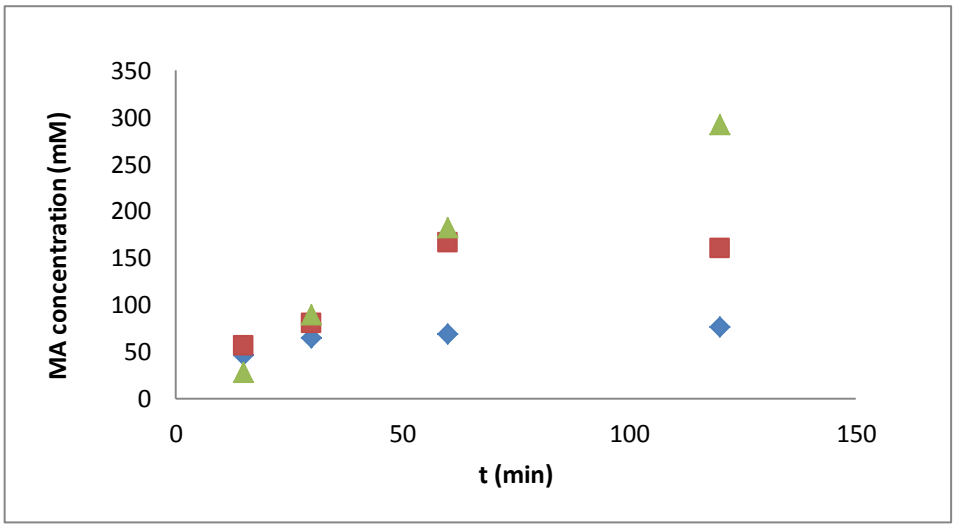
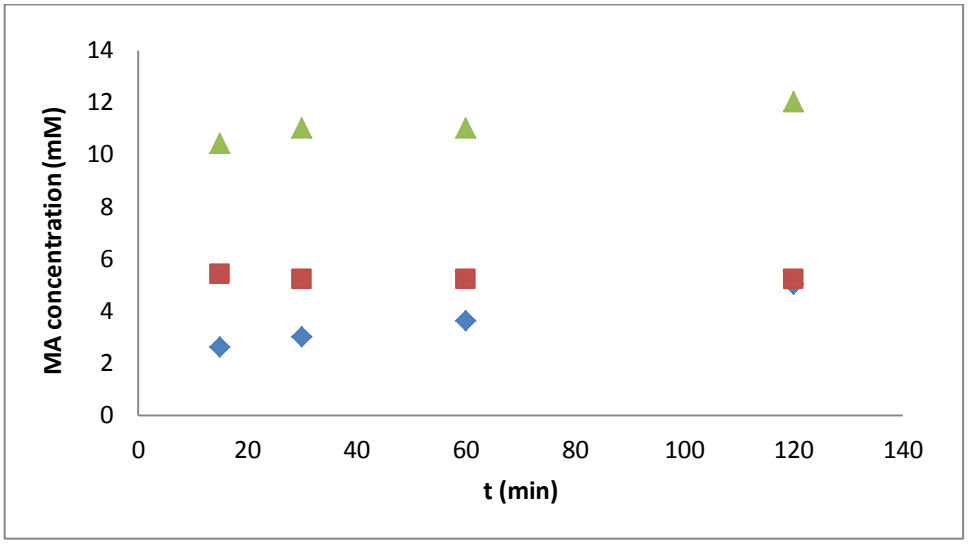


Figure 3.16: Biotransformation of 100 mM (diamond), 250 mM (square) or 500 mM (triangle) (*R,S*)-mandelonitrile at pH 5.0 (A) or 8.0 (B) in buffer:toluene (9:1) biphasic system by *E. coli* cells expressing nitrilase NitAb. The OD_{610} of the cell suspensions was ca. 9. The substrate was first dissolved in toluene and then added to the cell suspension; thus the resulting toluene portions were 9, 7 and 4 % in the 100, 250 and 500 mM reactions, respectively.

A



B

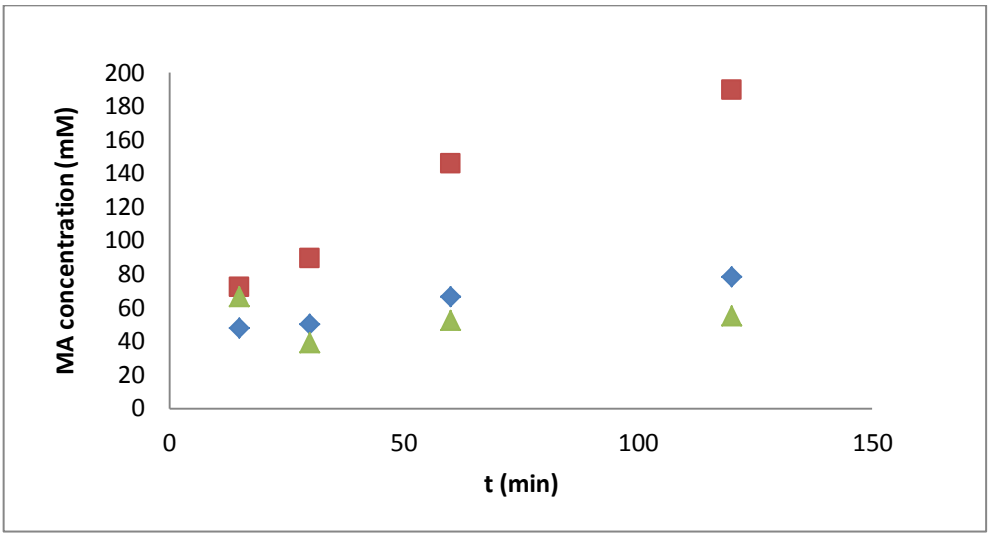


Figure 3.17: Biotransformation of 100 mM (diamond), 250 mM (square) or 500 mM (triangle) (*R,S*)-mandelonitrile at pH 5.0 (A) and 8.0 (B) in buffer:toluene (9:1) biphasic system by *E. coli* cells expressing nitrilase NitNh. The OD_{610} of the cell suspensions was ca. 9. The substrate was first dissolved in toluene and then added to the cell suspension; thus the resulting toluene portions were 9, 7 and 4 % in the 100, 250 and 500 mM reactions, respectively.

As a result of the first experiments, further biotransformations were carried out in 0.5 and 0.25 M (*R,S*)-mandelonitrile with nitrilase NitAb and NitNh, respectively.

The following reactions were performed at pH 5.0, 8.0, 9.0 and 10.0. Toluene formed 0, 10 or 20% of the reaction mixture and mandelonitrile was added separately. Milder shaking was applied to minimize the contact of the cells with the toluene layer.

Tables 3.7 and 3.8 summarize the effect of toluene and pH on the enantioselectivity of NitAb and NitNh and on mandelamide production by NitNh. The portion of mandelamide in the reactions of NitAb did not exceeed 2 % (molar percent of the total product). It is evident that the higher the pH, the better the enantioselectivity of both enzymes. The presence of 10 % toluene further increased the e.e. values, but at 20 % of toluene the enantioselectivity dropped again. In sum, by adding 10 % toluene and increasing pH to 10.0, the (*R*)-mandelic acid e.e. rose from 43 to 73 % in NitAb (conversion of 500 mM substrate) and from 73 to 97 % in NItNh (conversion of 250 mM substrate).

The increase in pH and addition of toluene also lowered the mandelamide production in NitNh (Table 3.8).

Table 3.7: The effect of pH and toluene content in the reaction mixture on enantioselectivity of NitAb and NitNh

pH	**(*R*)-mandelic acid e.e. (%) NitAb[a] / NitNh[b]**		
	No toluene	10 % toluene	20 % toluene
8.0	43 / 73	71 / 94	64 / 96
9.0	n. a.	69 / 97	65 / 96
10.0	n. a.	73 / 97	69 / 96

[a] conversion of 500 mM substrate

[b] conversion of 250 mM substrate

Table 3.8: The effect of pH and toluene content in the reaction mixture on the mandelamide production of NitNh

pH	NitNh[a] - mandelamide production (%)		
	No toluene	10 % toluene	20 % toluene
8.0	14.4	6.2	6.2
9.0	n. a.	4.1	4.4
10.0	n. a.	3.5	3.6

[a] conversion of 250 mM substrate

NitNh was from the pH 9.0 and higher highly selective for the (*R*)-mandelonitrile, and if it were not for the enzyme's instability at the substrate concentrations above 250 mM, it would be promising for the (*R*)-mandelic acid manufacture, as even this enzyme's tendency to produce amide could be lowered by the reaction conditions (presence of toluene, pH 9.0).

On the other hand, the moderate enantioselectivity of NitAb, almost no amide formation and its broad pH stability are promising for using this enzyme in the manufacture of (*S*)-mandelic acid from (*S*)-mandelonitrile under conditions of slow substrate racemization (pH 5.0).

Table 3.9: Batch transformations of (*R,S*)-mandelonitrile into (*R*)-mandelic acid or (*R,S*)-mandelic acid by NitAb and NitNh

Enzyme	Substrate conc. (mM)	Conversion (%)	Product concentration ($g\ L^{-1}$) / e.e. (%)	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Catalyst productivity ($g\ g_{dcw}^{-1}$)
NitAb[a]	100	98.4	14.7 / 0	88.2	7.4
NitNh[b]	250	85.7	32.5 / 96.5	195	14.7

[a] reaction conditions: pH 9, 10 % (v/v) toluene, 4 h

[b] reaction conditions: pH 5, 10 % (v/v) toluene, 4 h

The highest yields and productivities achieved in the enantioselective transformation of (*R*,*S*)-mandelonitrile by NitNh and the its non-selective transformation by NitAb are shown in Table 3.9. The transformations were carried out under optimized conditions (Table 3.9) in batch mode. After 4-h reaction at pH 5.0, NitAb transformed 100 mM (*R*,*S*)-mandelontrile with a 98 % conversion and a volumetric productivity of 88 g L^{-1} d^{-1}. After the same time, NitNh transforming 250 mM substrate reached 86% conversion, and a volumetric productivity of 195 g L^{-1} d^{-1}.

NitAn2 and NitNc showed better volumetric productivities than NitNh or NitAb at similar reaction conditions, being able to convert 500 mM substrate (for details see Appendix 3). However, the product e.e. was lower than in the experiments with low substrate concentrations (Petříčková *et al.*, 2012b). Therefore, the reaction volume was increased to 100 mL and the reaction mode was changed to fed-batch, applying multiple feeds of lower substrate amount.

First, NitAb, NitNh, NitAn2 and NitNc were tested in a reaction setup consisting of five feeds of 2.5 mmol (*R,S*)-mandelonitrile (one feed per hour). To increase the e.e. values, pH 10.0 was chosen to enable rapid substrate racemization. No toluene was added to exclude its adverse effect on the cells, as the larger reaction vessel (250-mL Erlenmeyer flask) would increase the contact between the organic and buffer layers.

Fig. 3.18 demonstrates the progress of each reaction. . In case of NitNh, the reaction proceeded slowly compared to the other three enzymes and even after being left to continue overnight, the final conversion was only 42.5 % (Tab. 3.10). This might be caused by the lower stability of NitNh at pH 10.0 (Veselá, 2011). In contrast, the reactions of NitAb, NitAn2 and NitNc continued after each feed, and after overnight incubation, the conversions were 90 % for NitAn2 and NitNc and 98 % for NitAb. The volumetric productivities were significantly lower than in batch mode (see above) because of lower substrate concentration. However, the e.e. values increased, namely for NitAn2 from 94.5 % in the batch mode to 97.6 % in fed-batch mode and NitNc from 95.2 % in the batch mode to 96.7 % in fed-batch mode (Tab. 3.10).

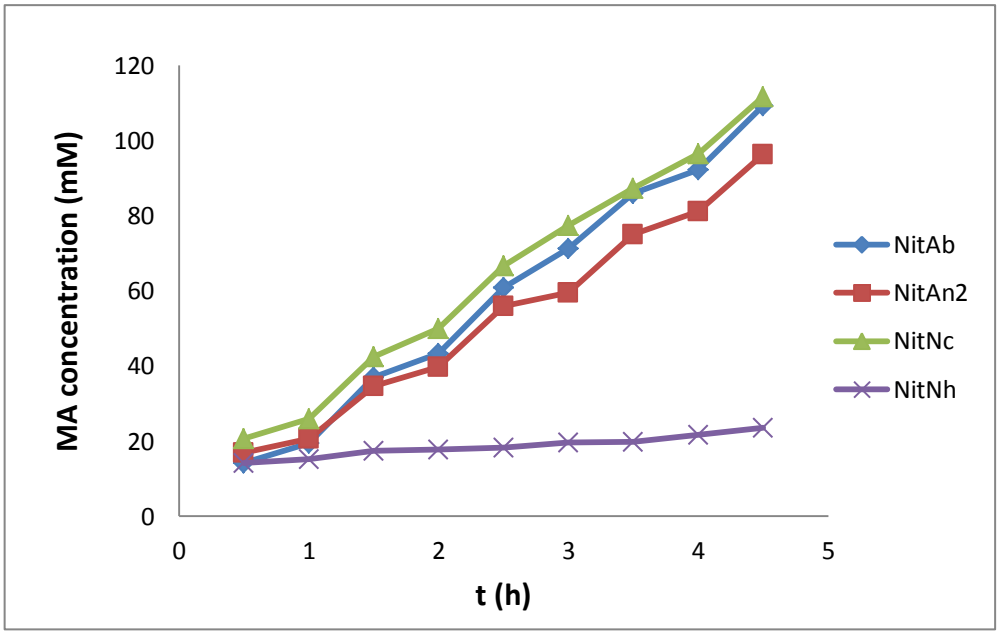


Figure 3.18: Fed-batch hydrolysis of (*R,S*)-mandelonitrile by NitAb (diamonds), NitAn2 (squares), NitNc (triangles) and NitNh (x). The reaction was performed in 250-mL Erlenmeyer flasks containing 100 mL of the cell suspension in 100 mM Gly/NaOH buffer of pH 10.0 and no toluene. The OD_{610} of the cell suspensions was 3.0-4.1. Feeds of 2.5 mmol of the substrate were added at the reaction start and then each hour.

Table 3.10: Fed-batch hydrolysis of (*R,S*)-mandelonitrile into (*R*)-mandelic acid by NitAb, NitAn2, NitNc and NitNh

Enzyme	Substrate amount (mmol)	Conversion (%)	Product concentration ($g\ L^{-1}$) / e.e. (%)	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Catalyst productivity ($g\ g_{dcw}^{-1}$)
NitAb	12.5	98.3	18.2 / 70.5	18.2	14.8
NitAn2	12.5	90.0	16.7 / 97.6	16.7	18.6
NitNc	12.5	90.6	15.5 / 96.7	15.5	13.4
NitNh	12.5	42.5	7.0 / 94.3	7.0	6.6

Owing to its highest e.e. and almost no mandelamide production, NitAn2 was selected for another fed-batch experiment with twelve feeds of 5 mmol (*R,S*)-mandelonitrile each hour.

After the total incubation time of 30.5 h, the reaction reached 90 % conversion of 12 x 0.67 g of (*R,S*)-mandelonitrile and the product concentration 77 g L^{-1}. The e.e. value, however, decreased to 95.6 %. This was probably caused by the higher amount of the substrate in the feeds (2.5 vs. 5 mmol). Compared to the batch mode of reaction, the volumetric productivities of the fed-batch experiments with NitAn2 were an order of magnitude lower. However, the catalyst productivity of the fed-batch was almost 5 times higher (see Table 3.11).

The catalyst productivity of NitAn2 in the 12 x 50 mM fed-batch was even 5-10 times higher than that of some of the bacterial arylacetonitrilases used in the conversions of high concentrations of mandelonitrile (Table 3.11).

Comparable experiments to those with NitAn2 were done with the arylacetonitrilase from *Alcaligenes* sp. (Zhang *et al.*, 2011). In the batch mode, the enzyme converted up to 500 mM mandelonitrile with volumetric productivity of 353 g L^{-1} d^{-1} and catalyst productivity 3.7 g g_{dcw}^{-1}. NitAn2 reached 571 g L^{-1} d^{-1} and 9 g g_{dcw}^{-1} at the same substrate concentration and experiment setup. In the fed-batch mode, catalyst productivity of *Alcaligenes* remained the same (Zhang *et al.*, 2010), an improvement was only achieved by cell-recycling in batch-mode with 100 mM substrate.

Table 3.11: Production of high concentrations of (*R*)-mandelic acid from (*R,S*)-mandelonitrile by bacterial nitrilases and the fungal nitrilase NitAn2

Enzyme source	Substrate concentration / reaction setup	Product concentration (g L^{-1}) / e.e. (%)	Volumetric productivity (g L^{-1} d^{-1})	Catalyst productivity (g g_{dcw}^{-1})	Ref.
Alcaligenes sp.	300–500 mM / batch	44-73 / 98.0	292-353	1.8-3.7	Zhang *et al.*, 2011
	5 x 100 mM / batch, cell recycling	70-80 / 98.0	55	55	
	6 x 100 mM / fed-batch	79 / n.a.	108	3.2	Zhang *et al.*, 2010
Alcaligenes faecalis	800 mM / fed-batch	105 / 99.0	337	8.4	Liu *et al.*, 2014
Burkholderia cenocepacia	6 x 500 mM / batch, cell recycling	50-73 / 95	982	156	Ni *et al.*, 2013
	1,000 mM / batch	150 / 97	895	60	
NitAn2	500 mM / batch	72 / 94.5	571	9	Veselá *et al.*, 2015
	12 x 50 mM / fed-batch	77 / 95.6	60	40	

3.4 Use of recombinant fungal nitrilases in the chemo-enzymatic synthesis of the taxol sidechain

The anti-cancer drug Paclitaxel (taxol) is a natural compound of a complex structure (Fig. 3.19). It was first isolated from the bark of the pacific yew (*Taxis brevifolia*). It is an antimicrotubule agent applied in the treatment of various cancer types, such as ovarian, gastric, head and neck, non-small lung, prostate and breast cancer (Nicolaou and Guy, 1995; Oettle, 2014; Lorusso *et al.*, 2014). In the cell, taxol binds to the polymerized microtubules, which leads to the disruption of the cell cycle and eventually to cell death (Magnani *et al.*, 2009; Sharma *et al.*, 2013). Nowadays, taxol is no longer isolated from the yew tree, but manufactured in a semi-synthetic way by coupling the N-benzoyl-(2*R*,3*S*)-3-phenylisoserine sidechain to the baccatin III core structure.

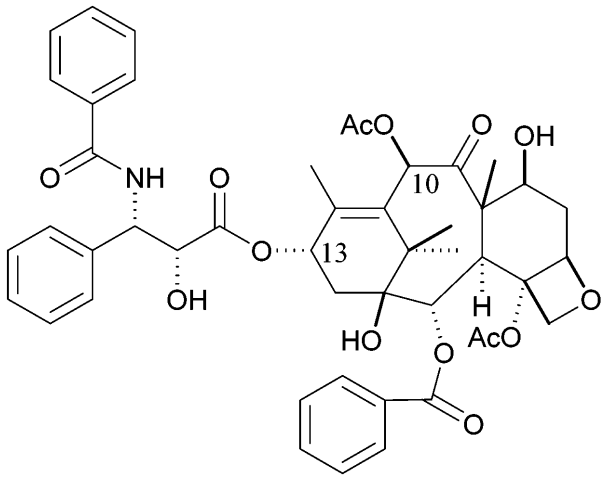


Figure 3.19: Structure of Paclitaxel (taxol), consisting of the baccatin III core structure and the (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine C13 sidechain.

Previously, different precursors of the C13 sidechain were prepared either by asymmetric chemical syntheses (Liu *et al.*, 2009; Dziedzic *et al.*, 2009; Qian *et al.*, 2010), or in a chemoenzymatic way using acylases (Cardillo *et al.*, 1999), lipases (Oshitari and Mandai, 2003) or reductases (Rimoldi *et al.*, 2011).

Here, nitrilases were employed for the first time in the chemoenzymatic synthesis of the sidechain precursor (Appendix 4).

The first step was the chemical synthesis of the dihydrooxazole (±)-*trans*-1 ((±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile (structure depicted in Fig. 3.20) from benzaldehyde (performed by Dipl.-Ing. Birgit Wilding, PhD. at the Institute of Organic Chemistry, Graz University of Technology). In the subsequent enzymatic step, the nitrile group of (±)-*trans*-1 was hydrolyzed to carboxylic acid either in a two-step reaction catalyzed by nitrile hydratase and amidase or in one-step nitrilase-catalyzed reaction (see Fig. 3.20). Commercial enzymes from Prozomix and Codexis (reactions with commercial enzymes performed by Dipl.-Ing. Birgit Wilding, PhD. at the Institute of Organic Chemistry, Graz University of Technology), as well as recombinant fungal arylacetonitrilases expressed in *E. coli* were employed in the hydrolytic step (for more information see Appendix 4). Here, only the results with fungal nitrilases will be further discussed.

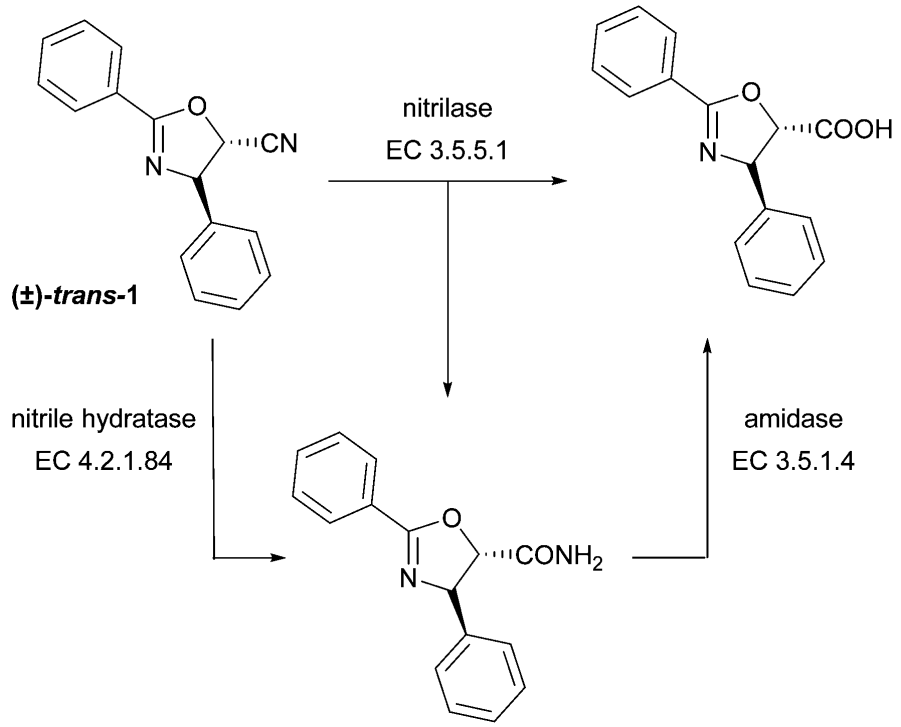


Figure 3.20: Enzymatic conversion of the taxol sidechain precursor (±)-*trans*-1 to the corresponding carboxylic acid and/or carboxamide.

The (±)-*trans*-1 compound was accepted as a substrate by all the tested fungal arylacetonitrilases, namely NitAb, NitAn2, NitAn3, NitAo, NitNc and NitNh.

Fig. 3.21 shows the conversions after 1h and 22 h of the reaction. Conversions of 30-100% were already observed within the first hour and complete conversions were achieved after 22 hours in all cases.

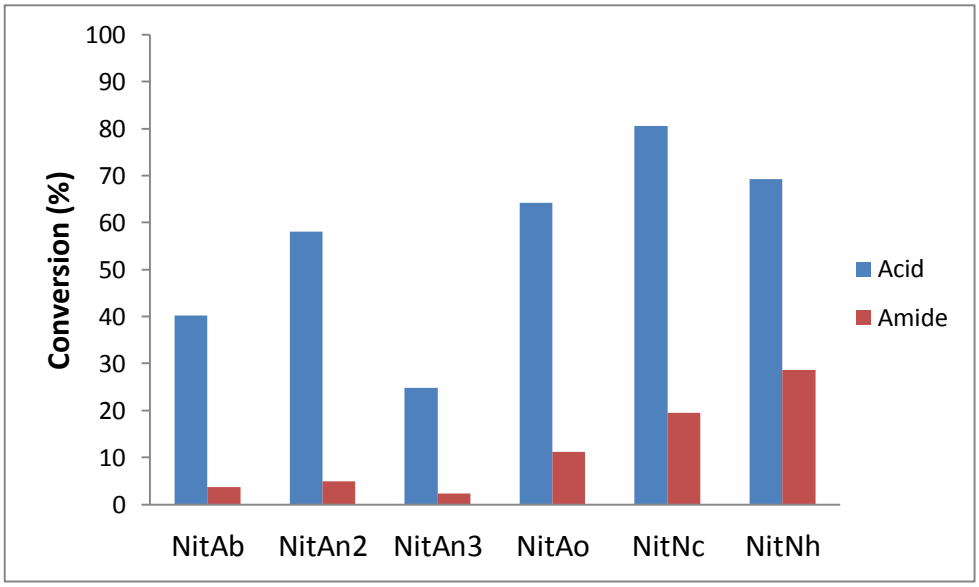
NitAo, NitNc and NitNh produced both acid and amide product throughout the reaction, whereas in NitAb, NitAn2 and NitAn3 the production of amide became prominent only in the late stages of the incubation. This was most evident for NitAn2, which only produced a minor portion of amide at the reaction beginning, but gave the one of the highest portions of amide after the complete conversion (similar as NitNh).

The e.e. values of the acid product of the nitrilase-catalyzed hydrolysis of (±)-*trans*-1 were in the moderate region (below 80 %). This can be explained by racemization and/or epimerization that could take place during the biotransformation itself.

The presence of the organic solvent in the reaction mixture with (±)-*trans*-1 should be investigated in more detail, as far as its effect on the e.e. values is concerned, as it was shown that organic solvents improved the activity and stereoselectivity of nitrilases (Layh and Willets, 1998; Kaul and Banerjee, 2008; Zhang et al, 2011; Vergne-Vaxelaire *et al.*, 2013). The effect of toluene on the enantioselectivity of nitrilases was also discussed in the previous section in context with the transformation of (*R*,*S*)-mandelonitrile.

NitNc displayed the highest activity for (±)-*trans*-1 of all the enzymes tested, and was used for the preparative scale production of the corresponding carboxylic acid.

A



B

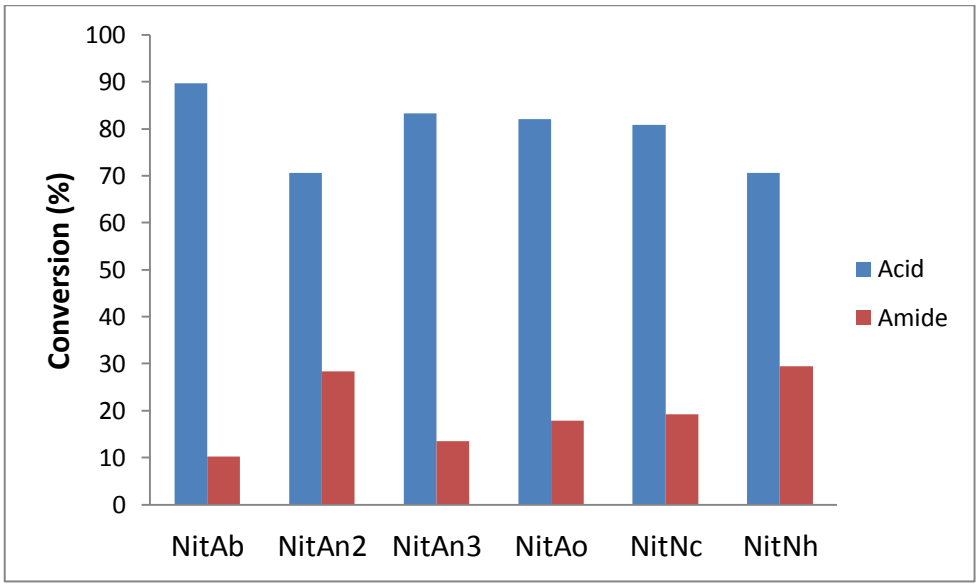


Figure 3.21: Biotransformation of 1.0 mM (±)-*trans*-1 (Fig. 3.21) into the corresponding carboxylic acid or amide by fungal arylacetonitrilases. A: after 1 h, B : after 22 h. The reaction mixtures consisted of the cell suspensions in 50 mM Tris/HCl buffer with 150 mM NaCl, pH 8.0, and (±)-*trans*-1 added from the stock solution in methanol to the final concentration of 1.0 mM. 500 mL samples were withdrawn from the reactions, mixed with 600 mL of methanol to inactivate the enzyme, centrifuged and analyzed by HPLC.

4 Conclusions

Nitrilases represent useful tools for the mild nitrile hydrolysis applicable in biotechnology. However, there are several hindrances that prevent their extensive use in chemical industry, such as their low stability and insufficient activity. Therefore, the search for new enzymes with improved properties is continued.

The effective, but time-consuming conventional screening based on the selection on special culture media was overcome by the high-throughput screening. These methods make use of spectrophotometric assays or pH indicator reactions.

Nevertheless, as the gene and protein databases expand, nowadays the most convenient method for obtaining new enzymes seems to be, apart from the metagenomic approach, the genome mining.

The amino acid sequences of fungal nitrilases discussed in this work were selected from the database using the sequences of previously studied enzymes as templates. According to the sequence similarities and presence of specific motifs in the vicinity of the catalytic cysteine, predictions could be made as to which substrate(s) the new recombinant enzymes prefer.

With one exception, the hypothesized substrate specificities of the new nitrilases were experimentally confirmed, and two new arylacetonitrilases, an aromatic nitrilase, a nitrilase of mixed-type substrate preference and two cyanide hydratases were identified.

For this work, recombinant fungal nitrilases NitAb and NitNh were purified and characterized. Both enzymes exhibited substrate specificities typical for arylacetonitrilases, that is a strong preference for phenylacetonitrile and (*R,S*)-mandelonitrile. Their kinetic parameters for these substrates were assessed. (*R,S*)-Mandelonitrile was hydrolyzed with (*R*)-selectivity in both cases; NitNh was highly enantioselective, whereas the enantioselectivity of NitAb was moderate. NitNh displayed a generally higher tendency to form amide as the reaction side-product than NitAb.

The temperature stabilities of both nitrilases were rather poor, however, both enzymes were highly stable at a broad range of pH (5.0-10.0).

Homology modeling, analytical centrifugation and electron microscopy revealed that both enzymes displayed the typical nitrilase α-β-β-α sandwich fold of the enzyme subunit. The subunits tend to form oligomeric structures: NitAb forms preferably dimers, tetramers, hexamers and dodecamers; the majority of the NitNh enzyme assembles into oligomeric structures of 12-24 subunits.

NitAb and NitNh were employed in the conversion of high concentrations of (*R,S*)-mandelonitrile. The effects of pH, toluene as a co-solvent and concentration of the substrate on the nitrilase activity, stability and enantioselectivity were studied. The presence of toluene as a co-solvent and higher pH values had a positive effect on enantioselectivity of both enzymes, although that of NitAb still remained in the moderate region.

In the next set of experiments, NitAb and NitNh together with NitAn2 and NitNc were tested in the conversion of (*R,S*)-mandelonitrile in a fed-batch mode. Of the four nitrilases tested, NitAn2 exhibited one of the highest conversions (90 %), and the highest e.e. values (up to 96.7 %) and catalyst productivity (up to 40 g g_{dcw}^{-1}), whereas its amide production was the lowest. In terms of the catalyst productivity, NitAn2 is comparable or even better than the bacterial arylacetonitrilases from the bacteria of the genus *Alcaligenes* previously employed in similar (*R,S*)-mandelonitrile conversions.

Thus, NitAb and NitAn2 were found potentially suitable for the application in enantiopure mandelic acid production as both enzymes form only a negligible portion of the amide by-product and are stable at high (500 mM) concentrations of the substrate. The low enantioselectivity and good stability at low pH renders NitAb a suitable candidate for an enantioretentive conversion of (*S*)-mandelonitrile to enantiopure (*S*)-mandelic acid. NitAn2, on the other hand, is highly (*R*)-selective, and therefore suitable for the manufacture of enantiopure (*R*)-mandelic acid from the racemic substrate.

NitAb, NitAn2, NitNc, NitNh, NitAn3 and NitAo were used in the production of a precursor of taxol, which is a well-known anti-cancer drug. To our knowledge, this was the first time nitrilases were employed in this reaction. All the nitrilases tested were able to fully convert 1.0 mM substrate and NitNc was sufficiently active to convert the substrate to the corresponding acid on a preparative scale.

In conclusion, this work confirmed that the genome mining and expression in *E. coli* is a suitable method for obtaining new fungal nitrilases. The expression of fungal nitrilase genes in *E. coli* is effective, as the nitrilase forms a major part of the cell protein and can be easily purified. New substrates were found that can be used for the nitrilase and cyanide hydratase activity assays, and the ability of the new nitrilases to transform industrially important compounds was verified.

5 References

Allen, J., Strobel, G. A.: The assimilation of $H^{14}CN$ by a variety of fungi. *Can J Microbiol*, **12**, 414 (1966)

Almatawah, Q. A., Cowan, D. A.: Thermostable nitrilase catalysed production of nicotinic acid from 3-cyanopyridine. *Enzyme Microb Technol*, 25, 718-724 (1999)

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J.: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, **25**, 3389- 3402 (1997)

Bandyopadhyay, A. K., Nagasawa, T., Asano, Y., Fujishiro, K., Tani, Y., Yamada, H.: Purification and characterization of benzonitrilases from *Arthrobacter* sp. strain J-1. *Appl Environ Microbiol*, **51**, 302-306 (1986)

Banerjee, A., Sharma, R., Banerjee, U. C.: The nitrile-degrading enzymes: current status and future prospects. *Appl Microbiol Biotechnol,* **60**, 33-44 (2002)

Banerjee, A., Dubey, S., Kaul, P., Barse, B., Piotrowski, M., Banerjee, U. C.: Enantioselective nitrilase from *Pseudomonas putida*: cloning, heterologous expression, and bioreactor studies. *Mol Biotechnol*, **41**, 35-41 (2009)

Barclay M., Tett V. A., Knowles C. J.: Metabolism and enzymology of cyanide/metallocyanide biodegradation by *Fusarium solani* under neutral and acidic conditions. *Enzyme Microb Technol*, **23**, 321-330 (1998)

Barglow, K. T., Saikatendu, K. S., Bracey, M. H., Huey, R., Morris, G. M., Olson, A. J., Stevens, R. C., Cravatt, B. F.: Functional proteomic and structural insights into molecular recognition in the nitrilase family enzymes. *Biochemistry*, **47**, 13514-13523 (2008)

Basheer, S., Kut, Ö. M., Prenosil, J. E., & Bourne, J. R.: Kinetics of enzymatic degradation of cyanide. *Biotechnol Bioeng*, **39**, 629-634 (1992)

Basile L. J., Willson R. C., Sewell B. T., Benedik M. J.: Genome mining of cyanide-degrading nitrilases from filamentous fungi. *Appl Microbiol Biotechnol*, **80**, 427-435 (2008)

Bauer, R., Hirrlinger, B., Layh, N., Stolz, A., Knackmuss, H. J.: Enantioselective hydrolysis of racemic 2-phenylpropionitrile and other (*R,S*)-2-arylpropionitriles by a new bacterial isolate, *Agrobacterium tumefaciens* strain d3. *Appl Microbiol Biotechnol*, **42**, 1-7 (1994)

Baxter J., Cummings S.P.: The current and future applications of microorganism in the bioremedation of cyanide contaminantion. *Antonie Van Leeuwenhoek*, **1**, 1-17 (2006)

Baxter, J., Garton, N. J., Cummings, S. P.: The impact of acrylonitrile and bioaugmentation on the biodegradation activity and bacterial community structure of a topsoil. *Folia Microbiol*, **51**, 591-597 (2006)

Bhalla, T. C., Miura, A., Wakamoto, A., Ohba, Y., Furuhashi, K.: Asymmetric hydrolysis of α-aminonitriles to optically active amino acids by a nitrilase of *Rhodococcus rhodochrous* PA-34. *Appl Microbiol Biotechnol*, **37**, 184-190 (1992)

Bhatia, R. K., Bhatia, S. K., Mehta, P. K., Bhalla, T. C.: Bench scale production of benzohydroxamic acid using acyl transfer activity of amidase from *Alcaligenes* sp. MTCC 10674. *J Ind Microbiol Biot*, **40**, 21-27 (2013).

Bjarnholt, N., Møller, B. L.: Hydroxynitrile glucosides. *Phytochemistry* **69**, 1947-1961 (2008)

Black, G. W., Brown, N. L., Perry, J. J., Randall, P. D., Turnbull, G., Zhang, M.: A high-throughput screening method for determining the substrate scope of nitrilases. *Chem Commun*, **51**, 2660-2662 (2015)

Brady, D., Beeton, A., Zeevaart, J., Kgaje, C., Van Rantwijk, F., Sheldon, R. A.: Characterisation of nitrilase and nitrile hydratase biocatalytic systems. *Appl Microbiol Biotechnol*, **64**, 76-85 (2004)

Brenner, C.: Catalysis in the nitrilase superfamily. *Curr Opin Struc Biol* **12**, 775-782 (2002)

Campos, M. G., Pereira, P., Roseiro, J. C.: Packed-bed reactor for the integrated biodegradation of cyanide and formamide by immobilised *Fusarium oxysporum* CCMI 876 and *Methylobacterium* sp. RXM CCMI 908. *Enzyme Microb Technol*, **38**, 848-854 (2006)

Cardillo, G., Tolomelli, A., Tomasini, C.: A New Diastereoselective Synthesis of anti-α-Alkyl α-Hydroxy β-Amino Acids. *Eur J Org Chem*, **1999**, 155-161 (1999)

Chmura, A., Rustler, S., Paravidino, M., van Rantwijk, F., Stolz, A., Sheldon, R. A.: The combi-CLEA approach: enzymatic cascade synthesis of enantiomerically pure (*S*)-mandelic acid. *Tetrahedron: Asymmetry*, **24**, 1225-1232 (2013)

Choi S. Y., Goo Y. M.: Hydrolysis of the nitrile group in α-aminophenylacetonitrile by nitrilase; Development of a new biotechnology for stereospecific production of S-α-phenylglycine. *Arch Pharm Res*, **9**, 45–47 (1986)

Cluness, M. J., Turner, P. D., Clements, E., Brown, D. T., O'Reilly, C.: Purification and properties of cyanide hydratase from *Fusarium lateritium* and analysis of the corresponding chy1 gene. *J Gen Microbiol*, **139**, 1807-1815 (1993)

Coady, T. M., Coffey, L. V., Catherine, O. R., Owens, E. B., Lennon, C. M.: A high throughput screening strategy for the assessment of nitrile-hydrolyzing activity towards the production of enantiopure β-hydroxy acids. *J Mol Catal B: Enzym*, **97**, 150-155 (2013)

Conn, E. E.: Biosynthesis of cyanogenic glycosides. *Naturwissenschaften* **66**, 28-34 (1979)

Conn, E. E.: Cyanogenic glycosides. In: Secondary Plant Products: A Comprehensive Treatise, 1st Edition, (Stumpf, W. and Conn, E. E. eds.) Academic Press, pp 479-498 (1981)

Crum, M. A., Park, J. M., Sewell, B. T., Benedik, M. J.: C-terminal hybrid mutant of *Bacillus pumilus* cyanide dihydratase dramatically enhances thermal stability and pH tolerance by reinforcing oligomerization. *J Appl Microbiol*, **118**, 881—889 (2015)

Davis, R. H., Nahrstedt, A.: Cyanogenesis in insects. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 11, (Kerkut, G. A. and Gilbert, L. I. eds.) Oxford: Pergamon Press, pp 635–654 (1985)

Dent, K. C., Weber, B. W., Benedik, M. J., Sewell, B. T.: The cyanide hydratase from *Neurospora crassa* forms a helix which has a dimeric repeat. *Appl Microbiol Biotechnol*, **82**, 271-278 (2009)

Duffey, S.S.: Cyanide and Arthropods. In: Cyanide in Biology, (Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J., Wissing, F. eds.) Academic Press, London, pp. 385–414 (1981)

Duran, R., Nishiyama, M., Horinouchi, S., Beppu, T.: Characterization of Nitrile Hydratase Genes Cloned by DNA Screeing from *Rhodococcus erythropolis*. *Biosci Biotech Bioch*, **57**, 1323-1328 (1993)

Dziedzic, P., Schyman, P., Kullberg, M., Córdova, A.: Highly Enantioselective Organocatalytic Addition of Aldehydes to N-(Phenylmethylene) benzamides: Asymmetric Synthesis of the Paclitaxel Side Chain and Its Analogues. *Chem Eur J*, **15**, 4044-4048 (2009)

Ebbs, S.: Biological degradation of cyanide compounds. *Curr Opin Biotech*, **15**, 231-236 (2004)

Effenberger, F., Osswald, S.: Enantioselective hydrolysis of (*R,S*)-2-fluoroarylacetonitriles using nitrilase from *Arabidopsis thaliana. Tetrahedron: Asymmetry*, **12**, 279-285 (2001)

Ema, T., Ide, S., Okita, N., Sakai, T.: Highly efficient chemoenzymatic synthesis of methyl (*R*)-*o*-chloromandelate, a key intermediate for clopidogrel, via asymmetric reduction with recombinant *Escherichia coli*. *Adv Synth Catal*, **350**, 2039–2044 (2008)

Endo, T., Watanabe, I.: Nitrile hydratase of *Rhodococcus* sp. N-774 purification and amino acid sequences. *FEBS Lett*, **243**, 61-64 (1989).

Fang, S., An, X., Liu, H., Cheng, Y., Hou, N., Feng, L., Huang, X., Li, C.: Enzymatic degradation of aliphatic nitriles by *Rhodococcus rhodochrous* BX2, a versatile nitrile-degrading bacterium. *Bioresour Technol*, **185**, 28-34 (2015)

Faull, J. L., Graeme-Cook, K. A., Pilkington, B. L.: Production of an isonitrile antibiotic by an UV-induced mutant of *Trichoderma harzianum*. *Phytochemistry*, **36**, 1273-1276 (1994)

Fernandes, B. C. M., Mateo, C., Kiziak, C., Chmura, A., Wacker, J., Van Rantwijk, F., Stolz, A., Sheldon, R. A.: Nitrile hydratase activity of a recombinant nitrilase. *Adv Synth Catal*, **348**, 2597-2603 (2006)

Fournand, D., Arnaud, A.: Aliphatic and enantioselective amidases: from hydrolysis to acyl transfer activity. *J Appl Microbiol*, **91**, 381-393 (2001)

Fournand, D., Bigey, F., Arnaud, A.: Acyl transfer activity of an amidase from *Rhodococcus* sp. strain R312: formation of a wide range of hydroxamic acids. *Appl Environ Microbiol* **64**, 2844-2852 (1998)

Fry, W. E., Munch, D. C.: Hydrogen cyanide detoxification by *Gloeocercospora sorghi*. *Physiol Plant Pathol*, **7**, 23-33 (1975)

Gavagan, J. E., Fager, S. K., Fallon, R. D., Folsom, P. W., Herkes, F. E., Eisenberg, A., Hann, E. C., DiCosimo, R.: Chemoenzymic production of lactams from aliphatic α, ω-dinitriles. *J Org Chem*, **63**, 4792-4801 (1998)

Gewitz, H. S., Pistorius, E. K., Voss, H. Venesland B.: Cyanide formation in preparations from *Chlorella* and New Zealand spinach leaves: effect of added aminoacids. *Planta* **131**, 149–153 (1976)

Goldlust, A., Bohak, Z.: Induction, purification, and characterization of the nitrilase of *Fusarium oxysporum* f. sp. *melonis*. *Biotechnol Appl Biochem*, **11**, 581–601 (1989)

Gong, J.-S., Lu, Z.-M., Li, H., Shi, J.-S., Zhou, Z.-M., Xu, Z.-H.: Nitrilases in nitrile biocatalysis: Recent progress and forthcoming research. *Microb Cell Fact* **11** (2012)

Gong, J. S., Lu, Z. M., Li, H., Zhou, Z. M., Shi, J. S., & Xu, Z. H.: Metagenomic technology and genome mining: emerging areas for exploring novel nitrilases. *Appl Microbiol Biotechnol*, **97**, 6603-6611 (2013)

Gupta, N., Balomajumder, C., Agarwal, V. K.: Enzymatic mechanism and biochemistry for cyanide degradation: A review. *J Hazard Mater* **176**, 1-13 (2010)

Harper, D. B.: Microbial metabolism of aromatic nitriles. Enzymology of CN cleavage by *Nocardia* sp. (*Rhodochrous* group) NCIB 11216. *Biochem J*, **165**, 309-319 (1977a)

Harper, D. B.: Fungal degradation of aromatic nitriles. Enzymology of CN cleavage by *Fusarium solani*. *Biochem J*, **167**, 685-692 (1977)

He, Y. C., Zhang, Z. J., Xu, J. H., Liu, Y. Y.: Biocatalytic synthesis of (*R*)-(−)-mandelic acid from racemic mandelonitrile by cetyltrimethylammonium bromide-permeabilized cells of *Alcaligenes faecalis* ECU0401. *J Ind Microbiol Biotechnol*, **37**, 741-750 (2010)

Hsu, J. C., Camper, N. D.: Degradation of ioxynil by soil fungus, *Fusarium solani*. *Soil Biol Biochem,* **11**, 19-22 (1979)

Ingvorsen K., Hojer-Pedersen B., Godfredsen S. E.: Novel cyanide-hydrolysing enzyme from *Alcaligenes xylosooxidans* subsp. *denitrificans*. *Appl Environ Microbiol*, **57**, 1783-1789 (1991)

Ingvorsen, K., Godtfredsen, S.E., Højer-Pedersen, B., Novo Nordisk. Microbial cyanide converting enzymes, their production and use. U.S. patent 5,116,744 (1992)

Jandhyala, D. M., Berman, M., Meyers, P. R., Sewell, B. T., Wilson, R. C., Benedik, M. J.: CynD, the cyanide dihydratase from *Bacillus pumilus*: Gene cloning and structural studies. *Appl Environ Microbiol* **69**, 4794-4805 (2003)

Jandhyala D. M., Willson R. C., Sewell B. T., Benedik M. J.: Comparison of cyanide-degrading nitrilases. *Appl Microbiol Biotechnol*, **68**, 327-335 (2005)

Janowitz, T., Trompetter, I., Piotrowski, M.: Evolution of nitrilases in glucosinolate-containing plants. *Phytochemistry*, **70**, 1680-1686 (2009)

Jenrich, R., Trompetter, I., Bak, S., Olsen, C. E., Møller, B. L., Piotrowski, M.: Evolution of heteromeric nitrilase complexes in *Poaceae* with new functions in nitrile metabolism. *Proc Natl Acad Sci*, **104**, 18848-18853 (2007)

Kaplan, O., Nikolaou, K., Pišvejcová, A., Martínková, L.: Hydrolysis of nitriles and amides by filamentous fungi. *Enzyme Microb Technol*, **38**, 260–4 (2006a)

Kaplan, O., Vejvoda, V., Charvátová-Pišvejcová, A., Martínková, L.: Hyperinduction of nitrilases in filamentous fungi. *J Ind Microbiol Biotechnol*, **33**, 891-896 (2006b)

Kaplan, O., Vejvoda, V., Plíhal, O., Pompach, P., Kavan, D., Bojarová, P., Bezouška, K., Macková, M., Cantarella, M., Jirků, V., Křen, V., Martínková, L.: Purification and characterization of a nitrilase from *Aspergillus niger* K10. *Appl Microbiol Biotechnol*, **73**, 567–75 (2006c)

Kaplan, O., Bezouška, K., Malandra, A., Veselá, A. B., Petříčková, A., Felsberg, J., Rinágelová, A., Křen, V., Martínková, L.: Genome mining for the discovery of new nitrilases in filamentous fungi. *Biotechnol Lett,* **33**, 309-312 (2011)

Kaplan, O., Veselá, A. B., Petříčková, A., Pasquarelli, F., Pičmanová, M., Rinágelová, A., Bhalla, T. C., Pátek, M., Martínková, L.: A comparative study of nitrilases identified by genome mining. *Mol Biotechnol* **54***,* 996-1003 (2013)

Kashiwagi, M., Fuhshuku, K. I., Sugai, T.: Control of the nitrile-hydrolyzing enzyme activity in *Rhodococcus rhodochrous* IFO 15564: preferential action of nitrile hydratase and amidase depending on the reaction condition factors and its application to the one-pot preparation of amides from aldehydes. *J Mol Catal B-Enzym*, **29**, 249-258 (2004)

Kato, Y., Tsuda, T., Asano, Y.: Nitrile hydratase involved in aldoxime metabolism from *Rhodococcus* sp. strain YH3-3. *Eur J Biochem*, **263**, 662-670 (1999)

Kaul, P., Banerjee, U. C.: Predicting enzyme behavior in nonconventional media: correlating nitrilase function with solvent properties. *J Ind Microbiol Biotechnol*, **35**, 713-720 (2008)

Ketterer, L., Keusgen, M.: Amperometric sensor for cyanide utilizing cyanidase and formate dehydrogenase. *Anal Chim Acta*, **673**, 54-59 (2010)

Keusgen, M., Kloock, J. P., Knobbe, D. T., Jünger, M., Krest, I., Goldbach, M., Klein, W., Schöning, M. J.: Direct determination of cyanides by potentiometric biosensors. *Sens Actuators, B*, **103**, 380-385 (2004)

Kikuchi, K.: Antibiotics from *Streptomyces* sp. no. E-212. Studies on *Streptomyces* antibiotics—XXIV. *J Antibiot, Ser A,* **8**, 145–147 (1955)

Kiziak, C., Conradt, D., Stolz, A., Mattes, R., Klein, J.: Nitrilase from *Pseudomonas fluorescens* EBC191: cloning and heterologous expression of the gene and biochemical characterization of the recombinant enzyme. *Microbiology*, **151**, 3639-3648 (2005)

Kiziak, C., Klein, J., Stolz, A.: Influence of different carboxy-terminal mutations on the substrate-, reaction-and enantiospecificity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. *Protein Eng, Des Sel*, **20**, 385-396 (2007)

Kiziak, C., Stolz, A.: Identification of amino acid residues responsible for the enantioselectivity and amide formation capacity of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191. *Appl Environ Microbiol*, **75**, 5592-5599 (2009)

Knowles, C. J., Bunch, A. W.: Microbial cyanide metabolism. *Adv Microb Physiol* **27**, 73–111 (1986)

Kobayashi, M., Shimizu, S.: Versatile nitrilases: Nitrile-hydrolysing enzymes. *FEMS Microbiol Lett*, **120**, 217-224 (1994)

Kobayashi, M., Goda, M., Shimizu, S.: Nitrilase catalyzes amide hydrolysis as well as nitrile hydrolysis. *Biochem Biophys Res Commun*, **253**, 662-666 (1998)

Kobayashi, M., Shimizu, S.: Metalloenzyme nitrile hydratase: structure, regulation, and application to biotechnology. *Nat Biotechnol*, **16**, 733-736 (1998)

Kohyama, E., Yoshimura, A., Aoshima, D., Yoshida, T., Kawamoto, H., Nagasawa, T.: Convenient treatment of acetonitrile-containing wastes using the tandem combination of nitrile hydratase and amidase-producing microorganisms. *Appl Microbiol Biotechnol*, ***72***, 600-606 (2006)

Kunz D. A., Wang C. S., Chen J. L.: Alternative routes of enzymatic cyanide metabolism in *Pseudomonas fluorescens* NCIMB 11764. *Microbiology*, **140**, 1705-1712 (1994)

Layh, N., Parratt, J., Willetts, A.: Characterization and partial purification of an enantioselective arylacetonitrilase from *Pseudomonas fluorescens* DSM 7155. *J Mol Catal B: Enzym*, **5**, 467-474 (1998)

Layh, N., Willetts, A.: Enzymatic nitrile hydrolysis in low water systems. *Biotechnol Lett*, **20**, 329-331 (1998)

Legras, J. L., Chuzel, G., Arnaud, A., Galzy, P.: Natural nitriles and their metabolism. *World J Microb Biot* **6**, 83-108 (1990)

Liese, A., Seelbach, K., Buchholz, A. and Haberland, J.: “Processes”. In: Industrial Biotransformations, (Liese, A., Seelbach, K. and Wandrey, C., eds.) Wiley VCH, Weinheim, pp 93–392 (2000)

Li, T., Liu, J., Bai, R., Ohandja, D. G., & Wong, F. S.: Biodegradation of organonitriles by adapted activated sludge consortium with acetonitrile-degrading microorganisms. *Water Res*, **41**, 3465-3473 (2007)

Li, H., Yang, T., Gong, J.-S., Xiong, L., Lu, Z.-M., Li, H., Shi, J.-S., Xu, Z.-H.: Improving the catalytic potential and substrate tolerance of *Gibberella intermedia* nitrilase by whole-cell immobilization. *Bioprocess Biosyst Eng*, **38**, 189–197 (2015)

Liu, W. J., Lv, B. D., Gong, L. Z.: An Asymmetric Catalytic Darzens Reaction between Diazoacetamides and Aldehydes Generates cis-Glycidic Amides with High Enantiomeric Purity. *Angew Chem*, **121**, 6625-6628 (2009)

Liu, Z. Q., Dong, L. Z., Cheng, F., Xue, Y. P., Wang, Y. S., Ding, J. N., Zheng, Y. G., Shen, Y. C.: Gene cloning, expression, and characterization of a nitrilase from *Alcaligenes faecalis* ZJUTB10. *J Agric Food Chem*, **59**, 11560-11570 (2011)

Lorusso, D., Petrelli, F., Coinu, A., Raspagliesi, F., Barni, S.: A systematic review comparing cisplatin and carboplatin plus paclitaxel-based chemotherapy for recurrent or metastatic cervical cancer. *Gynecol Oncol*, **133**, 117-123 (2014)

Maestracci, M., Thiery, A., Arnaud, A., Galzy, P.: A Study of the Mechanism of the Reactions Catalyzed by the Amidase *Brevibacterium* sp. R312. *Agr Biol Chem*, **50**, 2237-2241 (1986)

Magnani, M., Maccari, G., Andreu, J. M., Diaz, J. F., Botta, M.: Possible binding site for paclitaxel at microtubule pores. *FEBS J*, **276**, 2701-2712 (2009)

Mak, K. K., Law, A. W., Tokuda, S., Yanase, H., Renneberg, R.: Application of cyanide hydrolase from *Klebsiella* sp. in a biosensor system for the detection of low-level cyanide. *Appl Microbiol Biotechnol*, **67**, 631-636 (2005)

Malandra, A., Cantarella, M., Kaplan, O., Vejvoda, V., Uhnáková, B., Štěpánková, B., Kubáč, D., Martínková, L.: Continuous hydrolysis of 4-cyanopyridine by nitrilases from *Fusarium solani* O1 and *Aspergillus niger* K10. *Appl Microbiol Biotechnol*, **85**, 277-284 (2009)

Martínková, L., Křen, V.: Nitrile-and amide-converting microbial enzymes: stereo-, regio-and chemoselectivity. *Biocatal Biotransform*, **20**, 73-93 (2002)

Martínková, L., Vejvoda, V., Kaplan, O., Kubáč, D., Malandra, A., Cantarella, M., Bezouška, K., Křen, V.: Fungal nitrilases as biocatalysts: recent developments. *Biotechnol Adv*, **27**, 661-670 (2009)

Martínková, L., Křen, V.: Biotransformations with nitrilases. *Curr Opin Chem Biol*, **14**, 130-137 (2010)

Martínková, L.: Mining Genomes for Nitrilases. In: Cascade Biocatalysis: Integrating Stereoselective and Environmentally Friendly Reactions, (Riva, S., Fessner, W.-D. eds.) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp 271-282 (2014)

Martínková, L., Veselá, A. B., Rinágelová, A., Chmátal, M.: Cyanide hydratases and cyanide dihydratases: emerging tools in the biodegradation and biodetection of cyanide. *Appl Microbiol Biotechnol*, DOI: 10.1007/s00253-015-6899-0 (2015)

Mathew, C. D., Nagasawa, T., Kobayashi, M., Yamada, H.: Nitrilase-catalyzed production of nicotinic acid from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. *Appl Environ Microbiol*, **54**, 1030-1032 (1988)

Meyers P. R., Rawlings D. E., Woods D. R., Lindsey G. G.: Isolation and characterization of a cyanide dihydratase from *Bacillus pumilus* C1. *J Bacteriol*, **75**, 6105-6112 (1993)

Mueller, P., Egorova, K., Vorgias, C. E., Boutou, E., Trauthwein, H., Verseck, S., Antranikian, G.: Cloning, overexpression, and characterization of a thermoactive nitrilase from the hyperthermophilic archaeon *Pyrococcus abyssi*. *Protein Expression Purif*, **47**, 672-681 (2006)

Mylerová, V., Martínková, L.: Synthetic applications of nitrile-converting enzymes. *Curr Org Chem*, **7**, 1279-1295 (2003)

Nagasawa, T., Takeuchi, K., Yamada, H.: Characterization of a new cobalt-containing nitrile hydratase purified from urea-induced cells of *Rhodococcus rhodochrous* J1. *Eur J Biochem*, **196**, 581-589 (1991)

Nagasawa, T., Mauger, J., Yamada, H.: A novel nitrilase, arylacetonitrilase, of *Alcaligenes faecalis* JM3. *Eur J Biochem*, **194**, 765-772 (1990a)

Nagasawa, T., Nakamura, T., Yamada, H.: Production of acrylic acid and methacrylic acid using *Rhodococcus rhodochrous* J1 nitrilase. *Appl Microbiol Biotechnol*, **34**, 322–324. (1990b)

New Zealand Food Safety Authority: Cyanogenic glycosides – Information sheet. http://www.foodsafety.govt.nz/elibrary/industry/Cyanogenic_Glycosides-Toxin_Which.pdf

Ni, K., Wang, H., Zhao, L., Zhang, M., Zhang, S., Ren, Y., Wei, D.: Efficient production of (*R*)-(−)-mandelic acid in biphasic system by immobilized recombinant *E. coli*. *J Biotechnol*, **167**, 433-440 (2013)

Nicolaou, K. C., Guy, R. K.: The conquest of Taxol. *Angew Chem International Edition in English*, 34, 2079-2090 (1995)

Nolan, L. M., Harnedy P. A., Turner P., Hearne A. B., O'Reilly C.: The cyanide hydratase enzyme of *Fusarium lateritium* also has nitrilase activity. *FEMS Microbiol Lett*, **221**, 161-165 (2003)

Notredame, C., Higgins, D. G., Heringa, J.: T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol*, **302**, 205-17 (2000)

Oettle, H.: Progress in the knowledge and treatment of advanced pancreatic cancer: From benchside to bedside. *Cancer Treat Rev*, **40**, 1039-1047 (2014)

Oshitari, T., & Mandai, T.: Lipase-catalyzed kinetic resolution of 4-(N-Boc-amino)-1-alken-3-ols. *Synlett*, **15**, 2374-2376 (2003)

Osprian, I., Fechter, M. H., Griengl, H.: Biocatalytic hydrolysis of cyanohydrins: an efficient approach to enantiopure α-hydroxy carboxylic acids. *J Mol Catal B: Enzym*, **24**, 89-98 (2003)

O'Reilly, C., Turner, P. D.: The nitrilase family of CN hydrolysing enzymes – a comparative study. *J Appl Microbiol* **95**, 1161-1174 (2003)

Osswald, S., Wajant, H., Efenberger, F.: Characterization and synthetic applications of recombinant AtNIT1 from *Arabidopsis thaliana. Eur J Biochem*, **269**, 680-687 (2002)

Pace, H., C., Brenner, C.: The nitrilase superfamily: classification, structure and function. *Genome Biol,* **2** (2001)

Panova, A., Mersinger, L.J., Liu, Q., Foo, T., Roe, D.C., Spillan, W.L., Sigmund, A.E., Ben-Bassat, A., Wagner, L.W., O'Keefe, D.P., Wu, S., Petrillo, K.L., Payne, M.S., Breske, S.T., Gallagher, F.G., DiCosimo, R.: Chemoenzymatic synthesis of glycolic acid. *Adv Synth Catal*, **349**, 1462-1474 (2007)

Parker W. L., Rathnum M. L., Johnson J. H., Wells J. S., Principe P. A., Sykes R. B.: Aerocyanidin, a new antibiotic produced by *Chromobacterium violaceum*. *J Antibiot,* **41**, 454-460 (1988)

Payne, M. S., Wu, S., Fallon, R. D., Tudor, G., Stieglitz, B., Turner, I. M., Nelson, M. J.: A stereoselective cobalt-containing nitrile hydratase. *Biochemistry*, **36**, 5447-5454 (1997)

Peiser, G. D., Wang, T. T., Hoffman, N. E., Yang, S. F., Liu, H. W., Walsh, C. T.: Formation of cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to ethylene. *P Natl Acad Sci USA*, **81**, 3059-3063 (1984)

Petříčková, A., Veselá, A. B., Kaplan, O., Kubáč, D., Uhnáková, B., Malandra, A., Felsberg, J., Rinágelová, A., Weyrauch, P., Křen, V., Bezouška, K., Martínková, L.:

Purification and characterization of heterologously expressed nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* **93**, 1553-1561 (2012a)

Petříčková, A., Sosedov, O., Baum, S., Stolz, A., Martínková, L.: Influence of point mutations near the active site on catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa. J Mol Catal B: Enzym* **77**, 74-80 (2012b)

Petříčková, A.: Structure and biocatalytic properties of nitrilases from filamentous fungi. Dissertation, Faculty of Science, Charles University in Prague, pp 30-37 (2013)

Piotrowski, M., Schönfelder, S., Weiler, E. W.: The *Arabidopsis thaliana* isogene NIT4 and its orthologs in tobacco encode *β*-cyano-L-alanine hydratase/nitrilase. *J Biol Chem*, **276**, 2616-2621 (2001)

Piotrowski, M., Volmer, J. J.: Cyanide metabolism in higher plants: cyanoalanine hydratase is a NIT4 homolog. *Plant Mol Biol*, **61**, 111-122 (2006)

Piotrowski, M.: Primary or secondary? Versatile nitrilases in plant metabolism. *Phytochemistry* **69**, 2655-2667 (2008)

Podar, M., Eads, J. R., & Richardson, T. H.: Evolution of a microbial nitrilase gene family: a comparative and environmental genomics study. *BMC Evol Biol*, **5**, 42 (2005)

Prasad, S., Misra, A., Jangir, V. P., Awasthi, A., Raj, J., Bhalla, T. C.: A propionitrile-induced nitrilase of *Rhodococcus* sp. NDB 1165 and its application in nicotinic acid synthesis. *World J Microbiol Biotechnol*, **23**, 345-353 (2007)

Prasad, S., Bhalla, T. C.: Nitrile hydratases (NHases): at the interface of academia and industry. *Biotechnol Adv*, **28**, 725-741 (2010)

Přepechalová, I., Martínková, L., Stolz, A., Ovesná, M., Bezouška, K., Kopecký, J., Křen, V.: Purification and characterization of the enantioselective nitrile hydratase from *Rhodococcus equi* A4. *Appl Microbiol Biotechnol*, **55**, 150-156 (2001)

Qian, Y., Xu, X., Jiang, L., Prajapati, D., Hu, W.: A Strategy to Synthesize Taxol Side Chain and (−)-epi Cytoxazone via Chiral Brønsted Acid-Rh2 (OAc) 4 Co-catalyzed Enantioselective Three-Component Reactions†. *J Org Chem*, **75**, 7483-7486 (2010)

van Rantwijk, F., Stolz, A.: Enzymatic cascade synthesis of (S)-2-hydroxycarboxylic amides and acids: Cascade reactions employing a hydroxynitrile lyase, nitrile-converting enzymes and an amidase. *J Mol Catal B: Enzym*, **114**, 25-30 (2015)

Rausch, T., Hilgenberg, W.: Partial purification of nitrilase from Chinese cabbage. *Phytochemistry*, **19**, 747-750 (1980)

Rimoldi, I., Pellizzoni, M., Facchetti, G., Molinari, F., Zerla, D., Gandolfi, R.: Chemo-and biocatalytic strategies to obtain phenylisoserine, a lateral chain of Taxol by asymmetric reduction. *Tetrahedron: Asymmetry*, **22**, 2110-2116 (2011)

Rinágelová, A., Kaplan, O., Veselá, A. B., Chmátal, M., Křenková, A., Plíhal, O., Pasquarelli, F., Cantarella, M., Martínková, L.: Cyanide hydratase from *Aspergillus niger* K10: Overproduction in *Escherichia coli*, purification, characterization and use in continuous cyanide degradation. *Process Biochem*, **49**, 445-450 (2014)

Rinágelová, A.: Biotransformace anorganických a organických kyanidů. Diploma thesis, Faculty of Food and Biochemical Technology, UCT Prague, pp 49-50 (2013)

Robertson, D. E., Chaplin, J. A., DeSantis, G., Podar, M., Madden, M., Chi, E., Richardson, T., Milan, A., Miller, M., Weiner, D. P., Wong, K., McQuaid, J., Farwell, B., Preston, L. A., Tan, X. Q., Snead, M. A., Keller, M., Mathur, E., Kretz, P. L., Burk, M. J., Short, J. M.: Exploring nitrilase sequence space for enantioselective catalysis. *Appl Environ Microbiol*, **70**, 2429-2436 (2004)

Rustler, S., Motejadded, H., Altenbuchner, J., Stolz, A.: Simultaneous expression of an arylacetonitrilase from *Pseudomonas fluorescens* and a (*S*)-oxynitrilase from *Manihot esculenta* in *Pichia pastoris* for the synthesis of (*S*)-mandelic acid. *Appl Microbiol Biotechnol*, **80**, 87-97 (2008)

Saillenfait, A. M., Sabate, J. P.: Comparative developmental toxicities of aliphatic nitriles: In vivo and in vitro observations. *Toxicol Appl Pharmacol*, **163**, 149–163 (2000)

Sakai, N., Tajika, Y., Yao, M., Watanabe, N., Tanaka, I.: Crystal structure of hypothetical protein PH0642 from *Pyrococcus horikoshii* at 1.6 Å resolution. *Proteins: Struct, Funct, Bioinf*, **57**, 869-873 (2004)

Seffernick, J. L., Samanta, S. K., Louie, T. M., Wackett, L. P., Subramanian, M.: Investigative mining of sequence data for novel enzymes: A case study with nitrilases. *J Biotechnol* **143**, 17-26 (2009)

Sewell, B. T., Berman, M. N., Meyers, P. R., Jandhyala, D., Benedik, M. J.: The cyanide degrading nitrilase from *Pseudomonas stutzeri* AK61 is a two-fold symmetric, 14-subunit spiral. *Structure*, **11**, 1413-1422 (2003)

Sewell, B. T., Thuku, R. N., Zhang, X., Benedik, M. J.: Oligomeric tructure of nitrilases – effect of mutating interfacial residues on activity. *Ann NY Acad Sci*, **1056**, 153–159 (2005)

Sexton A. C., Howlet B. J.: Characterization of a cyanide hydratase gene in the phytopathogenic fungus *Leptosphaeria maculans*. *Mol Gen Genet*, **263**, 463-470 (2000)

Sharma, N. N., Sharma, M., Kumar, H., Bhalla, T. C.: *Nocardia globerula* NHB-2: Bench scale production of nicotinic acid. *Process Biochem*, **41**, 2078-2081 (2006)

Sharma, N. N., Sharma, M., & Bhalla, T. C.: An improved nitrilase-mediated bioprocess for synthesis of nicotinic acid from 3-cyanopyridine with hyperinduced *Nocardia globerula* NHB-2. *J Ind Microbiol Biotechnol*, **38**, 1235-1243 (2011)

Sharma, S., Lagisetti, C., Poliks, B., Coates, R. M., Kingston, D. G., Bane, S.: Dissecting Paclitaxel–Microtubule Association: Quantitative Assessment of the 2′-OH Group. *Biochemistry*, **52**, 2328-2336 (2013)

Shaw, N. M., Robins, K. T., Kiener, A.: Lonza: 20 Years of Biotransformations. *Adv Synth Catal*, **345**, 425–435 (2003)

Shen, M., Zheng, Y.-G., Shen, Y.-C.: Isolation and characterization of a novel *Arthrobacter nitroguajacolicus* ZJUTB06-99, capable of converting acrylonitrile to acrylic acid. *Process Biochem*, **44**, 781–785 (2009)

Singh, R., Sharma, R., Tewari, N., Rawat, D. S.: Nitrilase and its application as a 'green'catalyst. *Chem Biodiversity*, **3**, 1279-1287 (2006)

Sosedov, O., Matzer, K., Bürger, S., Kiziak, C., Baum, S., Altenbuchner, J., Chmura, A., van Rantwijk, F., Stolz, A.: Construction of Recombinant *Escherichia coli* Catalysts which Simultaneously Express an (*S*)-Oxynitrilase and Different Nitrilase Variants for the Synthesis of (*S*)-Mandelic Acid and (*S)*-Mandelic Amide from Benzaldehyde and Cyanide. *Adv Synth Catal*, **351**, 1531-1538 (2009)

Sosedov, O., Baum, S., Bürger, S., Matzer, K., Kiziak, C., Stolz, A.: Construction and application of variants of the *Pseudomonas fluorescens* EBC191 arylacetonitrilase for increased production of acids or amides. *Appl Environ Microbiol*, **76**, 3668-3674 (2010)

Sosedov, O., Stolz, A.: Random mutagenesis of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 and identification of variants, which form increased amounts of mandeloamide from mandelonitrile. *Appl Microbiol Biotechnol*, **98**, 1595-1607 (2014)

Stevenson, D., Feng, R., Dumas, F., Groleau, D., Mihot, A., Storer, A.: Mechanistic and structural studies on *Rhodococcus* ATCC 39484 nitrilase. *Biotechnol Appl Biochem* **15**, 283-302 (1992)

Straathof, A. J. J., Sie, S., Franco, T. T., van der Wielen, L. A. M.: Feasibility of acrylic acid production by fermentation. *Appl Microbiol Biotechnol*, **67**, 727–734 (2005)

Tani, Y., Kurihara, M., Nishise, H.: Characterization of nitrile hydratase and amidase, which are responsible for the conversion of dinitriles to mononitriles, from *Corynebacterium* sp. *Agric Biol Chem*, **53**, 3151-3158 (1989)

Tapper, B. A., MacDonald, M. A.: Cyanogenic compounds in culture of a psychrophylic basidiomycete (snow mold). *Can J Microbiol* **20**, 563–566 (1974)

Taylor, S. K., Chmiel, N. H., Simons, L. J., Vyvyan, J. R.: Conversion of hydroxy nitriles to lactones using *Rhodococcus rhodochrous* whole cells. *J Org Chem*, **61**, 9084-9085 (1996)

Thimann, K., Mahadevan, S.: Nitrilase II, its substrate specificity and possible mode of action. *Arch Biochem Biophys*, **107**, 62-68 (1964)

Thuku, R. N., Brady, S., Benedik, M. J., Sewell, B. T.: Microbial nitrilases: versatile, spiral forming, industrial enzymes. *J Appl Microbiol* **106**, 703-727 (2009)

Vejvoda, V., Kaplan, O., Klozova, J., Masák, J., Čejková, A., Jirků, V., Stloukal, R., Martínková, L.: Mild hydrolysis of nitriles by *Fusarium solani* strain O1. *Folia Microbiol*, **51**, 251-256 (2006a)

Vejvoda, V., Kaplan, O., Bezouška, K., Martínková, L.: Mild hydrolysis of nitriles by the immobilized nitrilase from *Aspergillus niger* K10. *J Mol Catal B: Enzym*, **39**, 55–8 (2006b)

Vejvoda, V., Kaplan, O., Kubáč, D., Křen, V., Martínková, L.: Immobilization of fungal nitrilase and bacterial amidase–two enzymes working in accord. *Biocatal Biotransform*, **24**, 414-418 (2006c)

Vejvoda, V., Šveda, O., Kaplan, O., Přikrylová, V., Elišáková, V., Himl, M., Kubáč, D., Pelantová, H., Kuzma, M., Křen, V., Martínková, L.: Biotransformation of heterocyclic dinitriles by *Rhodococcus erythropolis* and fungal nitrilases. *Biotechnol Lett*, **29**, 1119-1124 (2007)

Vejvoda, V., Kaplan, O., Bezouška, K., Pompach, P., Šulc, M., Cantarella, M., Benada, O., Uhnáková, B., Rinágelová, A., Lutz-Wahl, S., Fisher, L., Křen, V., Martínková, L.:

Purification and characterization of a nitrilase from *Fusarium solani* O1. *J Mol Catal B: Enzym*, **50**, 99-106 (2008)

Vejvoda, V., Martínková, L., Veselá, A. B., Kaplan, O., Lutz-Wahl, S., Fischer, L., Uhnáková, B.: Biotransformation of nitriles to hydroxamic acids via a nitrile hydratase–amidase cascade reaction. *J Mol Catal B: Enzym*, **71**, 51-55 (2011)

Vergne-Vaxelaire, C., Bordier, F., Fossey, A., Besnard-Gonnet, M., Debard, A., Mariage, A., ... & Zaparucha, A.: Nitrilase activity screening on structurally diverse substrates: Providing biocatalytic tools for organic synthesis. *Adv Synth Catal*, **355**, 1763-1779 (2013)

Veselá, A. B., Franc, M., Pelantová, H., Kubáč, D., Vejvoda, V., Šulc, M., Bhalla, T. C., Macková, M., Lovecká, P., Janů, P., Demnerová, K., Martínková, L.: Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity. *Biodegradation*, **21**, 761-770 (2010)

Veselá, A. B.: New possibilities of nitrilases in biocatalysis and bioremediation. Diploma thesis, Faculty of Science, Charles University in Prague, pp 49-53 (2011)

Veselá, A. B., Pelantová, H., Šulc, M., Macková, M., Lovecká, P., Thimová, M., Pasquarelli, F., Pičmanová, M., Pátek, M., Bhalla, T. C., Martínková, L.: Biotransformation of benzonitrile herbicides via the nitrile hydratase–amidase pathway in rhodococci. *J Ind Microbiol Biot*, **39**, 1811-1819 (2012)

Veselá, A. B., Křenková, A., Martínková, L.: Exploring the Potential of Fungal Arylacetonitrilases in Mandelic Acid Synthesis. *Mol biotechnol*, **57**, 466-474 (2015)

Vetter, J.: Plant cyanogenic glycosides. *Toxicon* **38**, 11-36 (2000)

Vorwerk, S., Biernacki, S., Hillebrand, H., Janzik, I., Müller, A., Weiler, E. W., Piotrowski, M.: Enzymatic characterization of the recombinant *Arabidopsis thaliana* nitrilase subfamily encoded by the NIT2/NIT1/NIT3-gene cluster. *Planta*, **212**, 508-516 (2001)

Wang P., VanEtten H. D.: Cloning and properties of a cyanide hydratase gene from the phytopathogenic fungus *Gloeocercospora sorghi*. *Biochem Biophys Res Commun*, **187**, 1048–1054 (1992)

Wang, P., Matthews, D. E., VanEtten, H. D.: Purification and characterization of cyanide hydratase from the phytopathogenic fungus *Gloeocercospora sorghi*. *Arch Biochem Biophys*, **298**, 569-575 (1992)

Wang, M. X., Li, J. J., Ji, G. J., Li, J. S.: Enantioselective biotransformations of racemic 2-aryl-3-methylbutyronitriles using *Rhodococcus* sp. AJ270. *J Mol Catal B: Enzym*, **14**, 77-83 (2001)

Wang, M. X., Feng, G. Q.: A novel approach to enantiopure cyclopropane compounds from biotransformation of nitriles. *New J Chem*, **26**, 1575-1583 (2002)

Wang, M. X., Wu, Y.: Nitrile biotransformations for the synthesis of enantiomerically enriched Baylis–Hillman adducts. *Org Biomol Chem*, **1**, 535-540 (2003)

Wang, M. X., Feng, G. Q., Zheng, Q. Y.: Synthesis of high enantiomeric purity gem-dihalocyclopropane derivatives from biotransformations of nitriles and amides. *Tetrahedron: Asymmetry*, **15**, 347-354 (2004)

Wang, H., Sun, H., Gao, W., Wei, D.: Efficient Production of (*R*)-*o*-Chloromandelic Acid by Recombinant *Escherichia coli* Cells Harboring Nitrilase from *Burkholderia cenocepacia* J2315. *Org Process Res Dev*, **18**,767–773 (2014)

Watanabe A., Yano K., Ikebukuro K., Karube I.: Cloning and expression of a gene encoding cyanidase from *Pseudomonas stutzeri* AK61. *Appl Microbiol Biotechnol*, **50**, 93-97 (1998)

Winkler, M., Kaplan, O., Vejvoda, V., Klempier, N., Martínková, L.: Biocatalytic application of nitrilases from *Fusarium solani* O1 and *Aspergillus niger* K10. *J Mol Catal B: Enzym*, **59**, 243-247 (2009)

Woodward, J. D., Weber, B. W., Scheffer, M. P., Benedik, M. J., Hoenger, A., Sewell, B. T.: Helical structure of unidirectionally shadowed metal replicas of cyanide hydratase from *Gloeocercospora sorghi*. *J Struct Biol*, **161**, 111-119 (2008)

Wu, Z. L., Li, Z. Y.: Enantioselective hydrolysis of various racemic α-substituted arylacetonitriles using *Rhodococcus* sp. CGMCC 0497.*Tetrahedron: Asymmetry*, **12**, 3305-3312 (2001)

Wu, S., Fogiel, A. J., Petrillo, K. L., Jackson, R. E., Parker, K. N., DiCosimo, R., Ben-Bassat, A., O'Keefe, D. P., Payne, M. S.: Protein engineering of nitrilase for chemoenzymatic production of glycolic acid. *Biotechnol Bioeng*, **99**, 717-720 (2008)

Xue, Y. P., Xu, M., Chen, H. S., Liu, Z. Q., Wang, Y. J., Zheng, Y. G.: A novel integrated bioprocess for efficient production of (*R*)-(−)-mandelic acid with immobilized *Alcaligenes faecalis* ZJUTB10. *Org Process Res Dev*, **17**, 213-220 (2013)

Yamamoto, K., Oishi, K., Fujimatsu, I., Komatsu, K.: Production of R-(-)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750. *Appl Environ Microbiol*, **57**, 3028-3032 (1991)

Yamamoto, K., Fujimatsu, I., Komatsu, K. I.: Purification and characterization of the nitrilase from *Alcaligenes faecalis* ATCC 8750 responsible for enantioselective hydrolysis of mandelonitrile. *J Ferment Bioeng*, **73**, 425-430 (1992).

Yanase H., Sakamoto A., Okamoto K., Kita K., Sato Y.: Degradation of the metal-cyano complex tetracyanonickelate (II) by *Fusarium oxysporum* N-10. *Appl Microbiol Biotechnol*, **53**, 328-334 (2000)

Zagrobelny, M., Bak, S., Moeller, B. L.: Cyanogenesis in plants and arthropods. *Phytochemistry*, **68**, 2757-2772 (2008)

Zagrobelny, M., Olsen, C. E., Pentzold, S., Fürstenberg-Hägg, J., Jørgensen, K., Bak, S., Møller, B. L., Motawia, M. S.: Sequestration, tissue distribution and developmental

transmission of cyanogenic glucosides in a specialist insect herbivore. *Insect Biochem Molec*, **44**, 44-53 (2014)

Zhang, Z. J., Xu, J. H., He, Y. C., Ouyang, L. M., Liu, Y. Y., Imanaka, T.: Efficient production of (*R*)-(−)-mandelic acid with highly substrate/product tolerant and enantioselective nitrilase of recombinant *Alcaligenes* sp. *Process Biochem*, **45**, 887-891 (2010)

Zhang, Z. J., Xu, J. H., He, Y. C., Ouyang, L. M., Liu, Y. Y.: Cloning and biochemical properties of a highly thermostable and enantioselective nitrilase from *Alcaligenes* sp. ECU0401 and its potential for (*R*)-(−)-mandelic acid production. *Bioprocess Biosyst Eng*, **34**, 315-322 (2011)

Zhang, C. S., Zhang, Z. J., Li, C. X., Yu, H. L., Zheng, G. W., & Xu, J. H.: Efficient production of (*R*)-*o*-chloromandelic acid by deracemization of *o*-chloromandelonitrile with a new nitrilase mined from *Labrenzia aggregata*. *Appl Microbiol Biotechnol*, **95**, 91-99 (2012)

Zhang, Z. J., Pan, J., Li, C. X., Yu, H. L., Zheng, G. W., Ju, X., Xu, J. H.: Efficient production of (*R*)-(−)-mandelic acid using glutaraldehyde cross-linked *Escherichia coli* cells expressing *Alcaligenes* sp. nitrilase. *Bioprocess Biosyst Eng*, **37**, 1241-1248 (2014a)

Zhang, L., Yin, B., Wang, C., Jiang, S., Wang, H., Yuan, Y. A., Wei, D.: Structural insights into enzymatic activity and substrate specificity determination by a single amino acid in nitrilase from *Syechocystis* sp. PCC6803. *J Struct Biol*, **188**, 93-101 (2014b)

Zhang, Z. J., Yu, H. L., Imanaka, T., Xu, J. H.: Efficient production of (*R*)-(−)-mandelic acid by isopropanol-permeabilized recombinant *E. coli* cells expressing *Alcaligenes* sp. nitrilase. *Biochem Eng J*, **95**, 71-77 (2015)

Zhu, D., Mukherjee, C., Biehl, E. R., Hua, L.: Discovery of a mandelonitrile hydrolase from *Bradyrhizobium japonicum* USDA110 by rational genome mining. *J Biotechnol*, **129**, 645-650 (2007)

Supplementary Data

```
CLUSTAL 2.1 multiple sequence alignment


NitGm           ----MSKSLKVAAIQAEPVWNDLQGGVNKSIGLIQEAAKEGANVIGYPEVFIPGYPWSIW 56
NitPc2          MTAPVERRVRVAAIQAEPVWNDLQGGVNKVISLLGDVGKEGANVVGFPEVFIPGYPWSIF 60
NitPm           ----MSKIVRVGAVQSEPVWLDLEGSVDKTISLIEKAAADGVNVLGFPEVWIPGYPWSMW 56
                    :.: ::*.*:*:**** **:*.*:* *.*: ... :*.**:*:***:*******::

NitGm           ANSPTENAPWINEYFKNSMEKESPEMDQIRAAVREAGVFVVLGYSERYRGTLYIAQSFID 116
NitPc2          TATPLDNAPFMEEYFHNSLAVDSDEMRRIQAAVKENGTFCVLGFSERYQGSLYISQVFIN 120
NitPm           TSAVINNSHIIHDYMNNSMRKDSPQMKRIQAAVKEAGMVVVLGYSERDGASLYMAQSFID 116
                : :  :*:  :.:*::**:  :* :* :*:***:* * . ***:***  .:**::* **:

NitGm           ETGTIVLHRRKIKPTHVERAIYGDGQGESLTNVADTKFGRVAGLNCWEHTQTLLRYYEYX 176
NitPc2          TDGQIVHHRRKTKPTHVERAYWGTGEGDSLKCVVDSPFGRIGGLNCWEHTQPLLRYYEYQ 180
NitPm           PSGEIVHHRRKIKPTHIERTIWGEGQAESLTCVIDSPFGKVGGLNCWEHLQPLLRYYEYS 176
                  * ** **** ****:**: :* *:.:**. * *: **::.******* *.*******

NitGm           QDVDIHVSSWPSIF--PQNVP-EWPYHITPECCKAFSHVVSMEGACFVLLASQIMTEENH 233
NitPc2          QDVDIHVASWPVLWDRPESVGSRWPYFITGDMSSRLSQVMAFEGTCFVLVCTQVMSEENF 240
NitPm           QGVQIHIASWPAEFEMPDPKKIAWLYHETGEASYRASQFFAIEGQAFVLVASQILTEANV 236
                *.*:**::***  :  *:     * *. * : .   *:..::** .***:.:*:::* *

NitGm           KKANVDGYDYTKKSGGGFSMIFSPFGEELVKPLAPNEEGILYADINLEEKYKAKQNLDIV 293
NitPc2          DKNKVRDVEHIQGTGGGFSAIFGPGGEPIAT-MPSDKEGILYANVDVNDKLRAKQWLDVV 299
NitPm           ERNNLTGNPVTKTPGGGFSMIFGPDGKPLCEPVDAGAEAILTADIDLRDIDKPKAFIDVV 296
                .: :: .    : .***** **.* *: :   : .. *.** *:::::.:  :.*  :*:*

NitGm           GHYSRPDQLSLRVNKHAAKPVFFANDL-- 320
NitPc2          GHYSRPDLLSLRVNTHPSKPVFFAEEPEK 328
NitPm           GHYARPDLLSLLVNPTVDKHVTTMKK--- 322
                ***:*** *** **    * *   :.
```

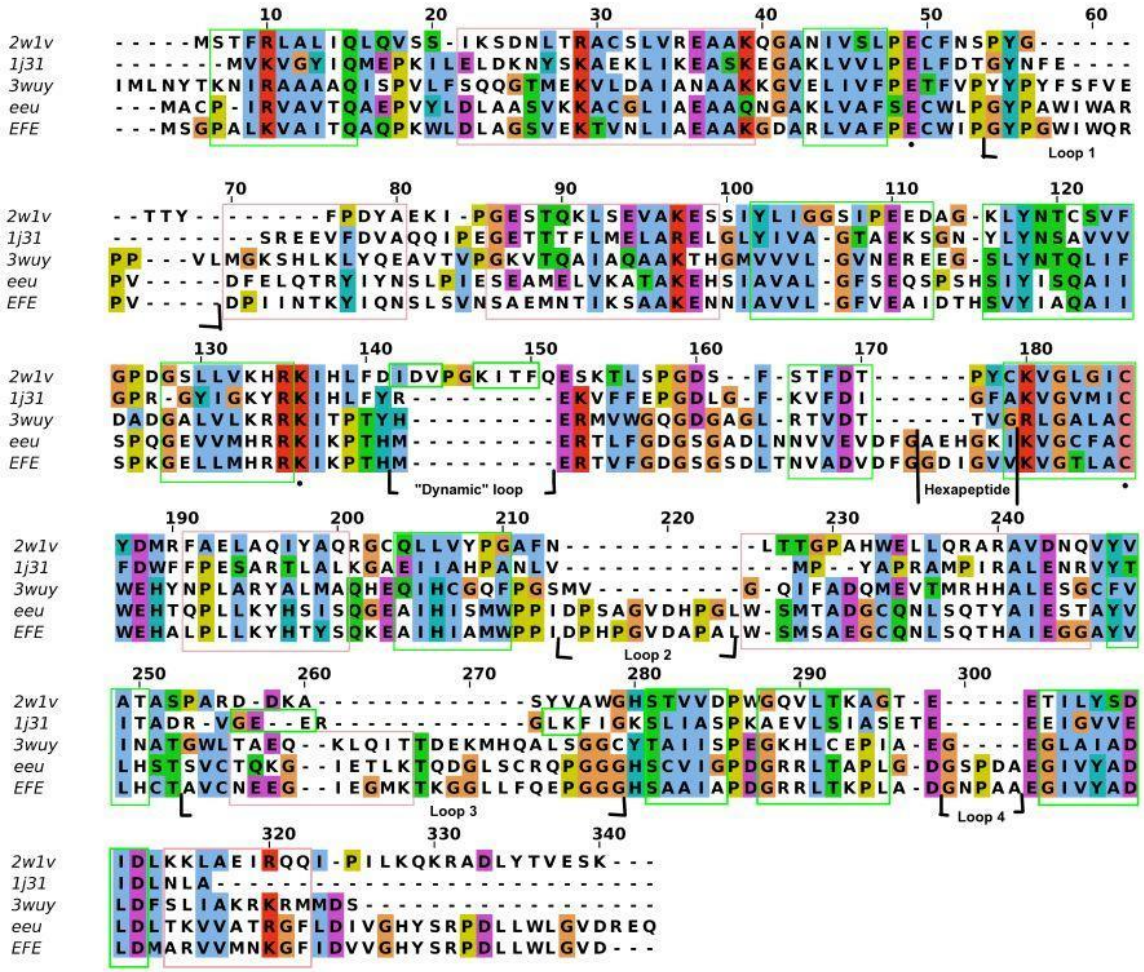
Supplementary figure S1: Multiple sequence alignment of the aromatic nitrilases from *Giberella moniliformis* (NitGm; GenBank: ABF83489), *Penicillium chrysogenum* Wisconsin 54-1255 (NitPc2; NCBI Reference Sequence: XP_002565836) and *Penicillium marneffei* ATCC18224 (NitPm; NCBI Reference Sequence: XP_002144951).

```
CLUSTAL 2.1 multiple sequence alignment


NitNc           ----MATTIKVAVTQAEPIWLDLQASIQKAVSLVHEAASNGAKIVAFSETWAPGYPGWCW 56
NitNh           ----MACPIRVAVTQAEPVYLDLAASVKKACGLIAEAAQNGAKLVAFSECWLPGYPAWIW 56
NitAb           ---MSGPALKVAITQAQPKWLDLAGSVEKTVNLIAEAAKGDARLVAFPECWIPGYPGWIW 57
NitAn3          ----MLSQVRVAVTQAEPVWLDLEATVKKTCDLIAEAAANGAQLVTFPECWIPGYPAWIW 56
NitAo           MTTPQPSQVRVAVTQAEPVWLDLKATVDKTCSLIAEAASKGAQLVSFPECWIPGYPAWIW 60
NitAn2          --MTASTKVRVAVTQHEPVWLDLHATVDKTCRLIAEAAGNGAQLITFPECWLPGYPAWIW 58
NitMg           ----MGAKVKVAVVQAEPVWFNLQETVKRVNELIELAYNKGAELIAFPEVFVPGYPTWIW 56
                        ::**:.* :* :::*  ::.:.  *:  *   .*.:::*.* : **** * *

NitNc           ARPVDPALNTKYAYNSLTANSPEMEQLQQAAKEDSIAVVIGFSERSSSGSLYIGQAIISP 116
NitNh           ARPVDFELQTRYIYNSLPIESEAMELVKATAKEHSIAVALGFSEQSPSHSIYISQAIISP 116
NitAb           QRPVDPIINTKYIQNSLSVNSAEMNTIKSAAKENNIAVVLGFVEAIDTHSVYIAQAIISP 117
NitAn3          ARPVDMRLSSIYIQNSLKIDSPQMASIQQCAAENKIVVVLGFSENL-HNSLYISQAIIAS 115
NitAo           TRPVDQELHSRYIQNSLTVSSPEMTQICKSANENNVIVVLGFSENI-HNSLYISQAIISN 119
NitAn2          CRPVDMGLFTTYLKNSLSYDSEHMRRICNAAAQHKITVVLGLSERD-GNSLYIGQCTIDS 117
NitMg           TNAADLDRNLMYTKNSLTYDSPEFISIIETVKKYPIHVVLGFSEKD-QGSLYISQCIIDN 115
                 ...*      *  ***  .*  :  :   . :  : *.:*: *     *:**.*. *

NitNc           QGEVALQRRKLKPTHMERTIFGDGSGPDLNCVAELDFGSELGSIKVGTLNCWEHAQPLLK 176
NitNh           QGEVVMHRRKIKPTHMERTLFGDGSGADLNNVVEVDFGAEHGKIKVGCFACWEHTQPLLK 176
NitAb           KGELLMHRRKIKPTHMERTVFGDGSGSDLTNVADVDFGGDIGVVKVGTLACWEHALPLLK 177
NitAn3          DGKILTTRKKIKPTHMERTIFGDSFGDCLQSVVDTSAG------RVGALSCWEHIQPLLK 169
NitAo           TGSILTTRKKIKATHMERTIFGDAFADCLDSVVETAVG------RVGALSCWEHIQPLLK 173
NitAn2          TGKIVMRRRKMKPTHMERTVFGESSGRSLLNVVDLPIG------KVGALACWEHIQPLLK 171
NitMg           TGEIVLKRRKFKPTHVERVIWGDTADSNMKSVVTLNFK-EAGPVEVGCLSCWEHMQPLLY 174
                 *.:   *:*:*.**:**.::*:     :  *.           .** : ****  ***

NitNc           FHEIQQGVVIHIAMWPPIDPYPGVEFPGLWSMTADGCQNLSQTFAVESGAFVLHCTAVCN 236
NitNh           YHSISQGEAIHISMWPPIDPSAGVDHPGLWSMTADGCQNLSQTYAIESTAYVLHSTSVCT 236
NitAb           YHTYSQKEAIHIAMWPPIDPHPGVDAPALWSMSAEGCQNLSQTHAIEGGAYVLHCTAVCN 237
NitAn3          YHTYAQREQIHVAAWPPLFPHS--EDGSLFSMSTEGTSSIARTYAIESQSFVLHTTTVIG 227
NitAo           YHTCAQREAIHVAAWPPLFEWGGPEDESLFSMSRDGTIALARTYAIESSSFVLHTTAVIS 233
NitAn2          YHTMIQGEEIHVSAWPVLHPHMG--GESLWGMSQEGGTGASQVYALESASFVLLTTAVLG 229
NitMg           YNSAAQHEKIHIGSWPALNDKD----LGVYCFTKAGFHGLARAYANQVQSFYLFT-SILG 229
                ::   *   **:. ** :         .:: ::  *    ::..* :  :: *   ::

NitNc           ESGIEAMDTRNGMVFREPGGGHSCVIGPDGRRLTQPLAD-KPSAEGIVYADLDLTRVVTN 295
NitNh           QKGIETLKTQDGLSCRQPGGGHSCVIGPDGRRLTAPLGDGSPDAEGIVYADLDLTKVVAT 296
NitAb           EEGIEGMKTKGGLLFQEPGGGHSAAIAPDGRRLTKPLADGNPAAEGIVYADLDMARVVMN 297
NitAn3          QSGIDRMATSTGALMSTPGGGCSAIFGPDGRQLSQPIPS---AEEGIIYADLDFEHIYHS 284
NitAo           QEGVEKMRTATGAIMNMPGGGSSAIFGPDGRLLSKPLLP---TEEGIIYADLEMHDIYKT 290
NitAn2          PTCVKKMNLSP--PWDTLGGGASAVIAPDGRRLTEPLPA---NEEGFVYADLDLDMILTC 284
NitMg           QRIQEALPDVKLSPYFEKGAGCGAVFAPDGSQITEDHPD---DFDGVIISELDMDKILLQ 286
                    . :           *.* .. :.***  ::          :*.: ::*::  :

NitNc           KSFQDIVGHYSRPDLLWLSYD----KEKK-----DAAVHRN------------ 327
NitNh           RGFLDIVGHYSRPDLLWLGVD----REQK-----ENIIAKQHKAAEQEAVQG- 339
NitAb           KGFIDVVGHYSRPDLLWLGVD----KAQK-----GCVVPKREPEQDV------ 335
NitAn3          KAFVDVCGHYSRPDLLWLGVEGGVKRHVR-----DNATTATP-QVEQQEEZ-- 329
NitAo           KAFVDVLGHYSRPDLLWLGVGSCDRRHVK-----EDAEERREDRVEVLZ---- 334
NitAn2          RHFVDACGHYSRPDLLWLGVDTREKTQHRPEGQADNAAYGLDVPSGLVEEEGA 337
NitMg           KNLVDIVGHYARPDMVSLSHN----RPNT------EFVNRKZ----------- 318
                : : *  ***:***:: *.
```

Supplementary figure S2: Multiple sequence alignment of the arylacetonitrilases from *Arthroderma benhamiae* CBS 112371 (NitAb; GenBank: EFE30690), *Aspergillus niger* CBS 513.88 (NitAn2; GenBank: CAK46742), *A. niger* CBS 513.88 (NitAn3; GenBank: CAK47246), *A. oryzae* RIB40 (NitAo; GenBank: BAE63579), *Neurospora crassa* OR74A (NitNc; GenBank: CAD70472), *Nectria haematococca* mpVI 77-13-4 (NitNh; GenBank: EEU45207) and the mixed-type nitrilase from *Meyerozyma guilliermondi* (*Pichia guilliermondi*) ATCC 6260 (NitMg; NCBI Reference Sequence: XP_001482890)

```
CLUSTAL 2.1 multiple sequence alignment


NitPc1          MVPVLKKYKAAAVNAEPGWFDLQESVRRTIHWIDEAGKAGCKLIAFPELWIPGYPYWAWK 60
NitAn1          MAPVLKKYKAAAVNAEPGWFNLEESVRRTIHWIDEAGKAGCKFIAFPELWIPGYPYWMWK 60
                *.******************:*:******************:************** **

NitPc1          VNYQESLPLLKKYRENSLPSDSDEMRRIREAAKANKIWVSLGYSELDLASLYTTQIMISP 120
NitAn1          VNYQESLPLLKKYRENSLPSDSDEMRRIRNAARANKIYVSLGYSEVDLASLYTTQVMISP 120
                ****************************:**:****:*******:*********:****

NitPc1          AGDVINHRRKIKATHVERLVFGDGTGDTTESVMDTEIGRIGHLNCWENMNPFLKAYAASL 180
NitAn1          SGDILNHRRKIRATHVERLVFGDGTGDTTESVIQTDIGRVGHLNCWENMNPFMKAYAASL 180
                :**::******:********************::*:***:************:*******

NitPc1          GEQVHIAAWPLYPGKETLKYPDPYTNVAEANADLVTPAYAIETGSFTLAPWQTITAEGIK 240
NitAn1          GEQVHVAAWPLYPGKETLKYPDPFTNVAEANADLVTPAYAIETGTYTLAPWQTITAEGIK 240
                *****:*****************:********************::**************

NitPc1          LNTPPGKELEDPNIYNGNGRIFGPDGQNLVPHPDKDFQGLLFVDIDLDEIHLTKSLADFG 300
NitAn1          LNTPPGKDLEDPHIYNGHGRIFGPDGQNLVPHPDKDFEGLLFVDIDLDECHLSKSLADFG 300
                *******:****:****:*******************:*********** **:*******

NitPc1          GHYMRPDLIRLLVDTNRKDLVVHEDRVNGGVAYTRTIDRVGLSAPLDASATEAQSESV 358
NitAn1          GHYMRPDLIRLLVDTNRKDLVVREDRVNGGVEYTRTVDRVGLSTPLDIANTVD-SEN- 356
                **********************:******** ****:******:*** : *   **.
```

Supplementary figure S3: Multiple sequence alignment of cyanide hydratases from *Aspergillus niger* K10 (CCF 3411) (NitAn1; GenBank: ABX75546) and *Penicillium chrysogenum* Wisconsin 54-1255 (NitPc1; NCBI Reference Sequence: XP_002562104)



Supplementary figure S4: Multiple sequence alignment of nitrilases from *Syechocystis* sp. PCC6803 (3wuy), Pyrococcus horikoshii (1j31), mouse (2w1v), *Arthroderma benhamiae* CBS 112371 (EFE) and *Nectria haematococca* mpVI 77-13-4 (Eeu). Conserved structural elements are marked by rectangulars: beta-strands – green; alpha-helices – pink. Catalytic triad is marked by black dots.

List of publications

Articles related to the presented thesis

Kaplan, O., **Veselá, A. B.**, Petříčková, A., Pasquarelli, F., Pičmanová, M., Rinágelová, A., Bhalla, T. C., Pátek, M., Martínková, L.: A comparative study of nitrilases identified by genome mining. *Mol Biotechnol* **54***,* 996-1003 (2013) **(Appendix 1)**

Veselá, A. B., Petříčková, A., Weyrauch, P., Martínková, L.: Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthroderma benhamiae*. *Biocatal Biotransform* **31**, 49-56 (2013) **(Appendix 2)**

Veselá, A. B., Křenková, A., Martínková, L.: Exploring the Potential of Fungal Arylacetonitrilases in Mandelic Acid Synthesis. *Mol Biotechnol*, **57**, 466-474 (2015) **(Appendix 3)**

Wilding, B., **Veselá, A. B.**, Perry, J. J., Black, G. W., Zhang, M., Martínková, L., Klempier, N.: An investigation of nitrile transforming enzymes in the chemo-enzymatic synthesis of the taxol sidechain. *Org Biomol Chem*, **13**, 7803-7812 (2015) **(Appendix 4)**

Other research articles

Martínková, L., Pátek, M., **Veselá, A. B.**, Kaplan, O., Uhnáková, B., Nešvera, J.: Catabolism of nitriles in *Rhodococcus*. In: Biology of *Rhodococcus*, (Alvarez, H. M. ed.) Springer Berlin Heidelberg, pp. 171-206 (2010)

Veselá, A. B., Franc, M., Pelantová, H., Kubáč, D., Vejvoda, V., Šulc, M., Bhalla, T. C., Macková, M., Lovecká, P., Janů, P., Demnerová, K., Martínková, L.: Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity. *Biodegradation*, **21**, 761-770 (2010)

Kaplan, O., Bezouška, K., Plíhal, O., Ettrich, R., Kulik, N., Vaněk, O., Kavan, D., Benada, O., Malandra, A., Šveda, O., **Veselá, A. B.**, Rinágelová, A., Slámová, K., Cantarella, M., Felsberg, J., Dušková, J., Dohnálek, J., Kotik, M., Křen, V., Martínková, L.: Heterologous expression, purification and characterization of nitrilase from *Aspergillus niger* K10. *BMC Biotechnol.* **11**, 2 (2011)

Kaplan, O., Bezouška, K., Malandra, A., **Veselá, A. B.**, Petříčková, A., Felsberg, J., Rinágelová, A., Křen, V., Martínková, L.: Genome mining for the discovery of new nitrilases in filamentous fungi. *Biotechnol. Lett.* **33**, 309-312 (2011)

Vejvoda, V., Martínková, L., **Veselá, A. B.**, Kaplan, O., Lutz-Wahl, S., Fischer, L., Uhnáková, B.: Biotransformation of nitriles to hydroxamic acids via a nitrile hydratase–amidase cascade reaction. *J Mol Catal B: Enzym*, **71**, 51-55 (2011)

Petříčková, A., **Veselá, A. B.**, Kaplan, O., Kubáč, D., Uhnáková, B., Malandra, A., Felsberg, J., Rinágelová, A., Weyrauch, P., Křen, V., Bezouška, K., Martínková, L.: Purification and characterization of heterologously expressed nitrilases from filamentous fungi. *Appl Microbiol Biotechnol* **93**, 1553-1561 (2012)

Veselá, A. B., Pelantová, H., Šulc, M., Macková, M., Lovecká, P., Thimová, M., Pasquarelli, F., Pičmanová, M., Pátek, M., Bhalla, T. C., Martínková, L.: Biotransformation of benzonitrile herbicides via the nitrile hydratase–amidase pathway in rhodococci. *J Ind Microbiol Biot*, **39**, 1811-1819 (2012)

Rinágelová, A., Kaplan, O., **Veselá, A. B.**, Chmátal, M., Křenková, A., Plíhal, O., Pasquarelli, F., Cantarella, M., Martínková, L.: Cyanide hydratase from *Aspergillus niger* K10: Overproduction in *Escherichia coli*, purification, characterization and use in continuous cyanide degradation. *Process Biochem*, **49**, 445-450 (2014)

Martínková, L., **Veselá, A. B.**, Rinágelová, A., Chmátal, M.: Cyanide hydratases and cyanide dihydratases: emerging tools in the biodegradation and biodetection of cyanide. *Appl Microbiol Biotechnol*, DOI: 10.1007/s00253-015-6899-0 (2015)

Oral presentations

Veselá, A. B.: New fungal and bacterial nitrilases for biocatalysis and bioremediation. COST Action CM0701 "CASCAT" Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry – A Joint Workshop – Conference, Vilnius, Lithuania (8. – 11.9.2010)

Veselá, A. B.: Heterologous expression and characterization of novel fungal arylacetonitrilases. Annual seminar of project GACR 305/09/H008 „Preparation, biotransformation and optimalization of compounds with antitumour and antimicrobial effects", Nové Hrady (9. – 11.6.2011)

Veselá, A. B.: Novel recombinant fungal nitrilases and cyanide hydratases and their application in biotransformation of mandelonitrile and HCN. World Biotechnology Congress 2013, Boston, USA (3. – 6.6. 2013)

Poster presentations

Veselá, A. B., Kubáč, D., Vejvoda, V., Martínková, L.: Hydrolysis of benzonitrile herbicides by soil actinobacteria. 62. Sjezd asociací českých a slovenských chemických společností (28. - 30.6.2010)

Veselá, A. B., Martínková, L., Kaplan, O., Petříčková, A., Malandra, A. Rinágelová, A. Benada, O., Křen, V., Bezouška, K.: Purification and characterization of recombinant nitrilase from *Gibberella moniliformis* co-expressed with GroES/EL chaperone system. 5th International Congress on Biocatalysis, Hamburg, Germany (29.8.2010 – 2.9.2010)

Veselá, A. B., Weyrauch, P., Petříčková, A., Rinágelová, A., Martínková, L.: Heterologous expression and characterization of novel fungal arylacetonitrilases. Biotrans 2011, Giardini Naxos, Sicily, Italy (2. – 6.10.2011)

Veselá, A. B., Weyrauch, P., Petříčková, A., Rinágelová, A., Martínková, L.: Recombinant fungal arylacetonitrilases in mandelonitrile biotransformation. Multistep enzyme-catalyzed processes, Graz, Austria (10. – 13.4.2012)

Kaplan, O., Petříčková, A., **Veselá, A. B.**, Rinágelová, A., Martínková, L.: Recombinant nitrilases from various microorganisms – an in-depth substrate specificity screening. 6th International Congress on Biocatalysis, Hamburg, Germany (2. – 6.9.2012)

Rinágelová, A., **Veselá, A. B.**, Kaplan, O., Chmátal, M., Petříčková, A., Pasquarelli, F., Cantarella, M., Martínková, L.: Cyanide bioremediation using novel fungal cyanide hydratases. Biotrans 2013, Manchester, UK (21. – 25.7.2013)

Veselá, A. B., Martínková, L., Rinágelová, A., Rucká, L., Kaplan, O.: Substrate specificity of fungal nitrilases and cyanide hydratases can be predicted from their sequences. 4th international conference on Novel Enzymes, Ghent, Belgium (14. – 17.10.2014)

Appendices – related published articles

Appendix 1

Kaplan, O., **Veselá, A. B.**, Petříčková, A., Pasquarelli, F., Pičmanová, M., Rinágelová, A., Bhalla, T. C., Pátek, M., Martínková, L.: A comparative study of nitrilases identified by genome mining. *Mol Biotechnol* **54***,* 996-1003 (2013)

Appendix 2

Veselá, A. B., Petříčková, A., Weyrauch, P., Martínková, L.: Heterologous expression, purification and characterization of arylacetonitrilases from *Nectria haematococca* and *Arthroderma benhamiae*. *Biocatal Biotransform* **31**, 49-56 (2013)

Appendix 3

Veselá, A. B., Křenková, A., Martínková, L.: Exploring the Potential of Fungal Arylacetonitrilases in Mandelic Acid Synthesis. *Mol Biotechnol*, **57**, 466-474 (2015)

Appendix 4

Wilding, B., **Veselá, A. B.**, Perry, J. J., Black, G. W., Zhang, M., Martínková, L., Klempier, N.: An investigation of nitrile transforming enzymes in the chemo-enzymatic synthesis of the taxol sidechain. *Org Biomol Chem*, **13**, 7803-7812 (2015)